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Special Problems of Experimenting *in ovo* on the Early Chick Embryo, and a Solution

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INTRODUCTION

IT seems to be generally accepted that experimenting *in ovo* on the chick during the early stages of development (up to about 48 hours) is fraught with the greatest difficulty. After about this time no serious technical problems arise and a high proportion of successful results can be expected. It is natural to ask why there should be this change-over from extreme difficulty to reasonable simplicity. New (1955) attributed to this 'inaccessibility of the chick embryo in the egg' the invention of his own and many other *in vitro* methods during the last 30 years. There is no doubt that, when short-term experiments only are required, *in vitro* methods will probably always be preferred. But all *in vitro* methods suffer from the disadvantage that the embryo cannot be expected to survive for more than 48 hours or so after explantation. There are many experiments, however, in which operative interference is required at stages of up to about 12 somites, and in which it is necessary for the embryo to develop thereafter for a considerably longer time.

Grabowski (1956) gives some important technical details for operating *in ovo*. His method depends upon two main steps: (1) only a very small hole, 0·1–0·2 mm. in diameter, is made in the vitelline membrane, and (2) the air space overlying the blastoderm is topped up with saline or watery albumen before replacing the window in the shell. This step, i.e. topping up, he describes as 'absolutely essential' to prevent drying of the exposed surface of the blastoderm. The egg is now rolled so that the blastoderm with its overlying vitelline membrane is faced with intact shell membrane. This is a very satisfactory method, but suffers from the disadvantage that, for many purposes, a hole of this size in the vitelline membrane is prohibitively small. It is extremely difficult to do much with a needle through the hole without tearing the membrane (which even when stained with Nile blue sulphate quickly loses the stain and is difficult to see) and thus accidentally enlarging the opening. Furthermore, if a

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large extirpation is undertaken, it may not be possible to extract the fragment through such a small hole. One of the primary aims of the present investigation has therefore been to find a means of making a large opening in the vitelline membrane without prejudice to the embryo's survival.

In a previous communication (Silver, 1959) the role of the vitelline membrane in the production of abnormalities affecting the embryo and of the amnion was described. One of the main causes of trouble was the drying of the vitelline membrane with subsequent sticking to the embryo, or to its membranes, or to the expanding blastoderm. The mechanism of surface drying was thought to be related to the secretion of sub-blastodermal fluid, for New (1956) has shown that the blastoderm secretes fluid downwards into the yolk. This fluid is drawn from the albumen which overlies the blastoderm. Evidently it is this layer of albumen which is normally responsible for keeping the vitelline membrane moist, but even in the unoperated egg it is extremely thin. The point which arises from New's work, and which needs special emphasis here, is that we are dealing with a continuous process. Thus it will not be sufficient to prevent drying only while the actual experiment is being performed; dehydration of the supra-blastodermal fluid will continue during the later stages of development. What is needed is a method of replenishing the suprablastodermal fluid with fresh albumen *after* the egg has been returned to the incubator. 'Topping up' achieves this end but is inapplicable in the presence of a large opening in the vitelline membrane, for reasons which will be explained below.

When the normal experimental procedure is adopted, as soon as the window is cut in the shell membrane the blastoderm drops down, an air space appears, and, instead of being convex upwards as it normally is, the blastoderm becomes concave upwards. Under these conditions it is now possible to make a really large opening in the membrane exposing the whole of the area pellucida, without any tendency for herniation to occur, provided the embryo is orientated immediately below the centre of the shell window. If now, to prevent subsequent drying, we employ the Grabowski method and top up the egg, we find that as soon as the blastoderm regains its normal shape, becoming convex upwards once more, the embryo and surrounding blastoderm begin to spill out through the opening in the vitelline membrane.

We now seem to have reached an impasse. If we make an opening in the vitelline membrane large enough to permit major operative procedures, herniation will occur when we top up. If we do not top up, on the other hand, surface drying will ensue and adhesion between the embryo or its membranes and the vitelline membrane will take place, and the expansion of the area vasculosa will be arrested or very seriously retarded.

Silver (1957) devised one method of escaping from this difficult position by repairing the opening in the vitelline membrane with a single silk thread. This method suffers from the disadvantage, however, of being somewhat laborious; it may take up to 15 minutes. In fact, it is technically much more difficult than

experimenting on the embryo itself. A search was therefore undertaken to find a new technique capable of preventing drying without topping up.

In all, about 1,500 investigations have been carried out. It is not proposed to describe more than a small fraction of these, i.e. those which seem immediately relevant to the final method.

METHODS

Operating incubator

These experiments proceeded in a specially constructed operating incubator, connected to a suitable air-conditioning plant, in which the temperature was maintained at 99·5° F. and the humidity as near 100 per cent. as possible in order to prevent evaporation during the actual experiment. It was possible to observe the embryo for long periods, see what really did go wrong, and devise appropriate preventive measures. This apparatus was made of transparent perspex with double walls within which, in the roof, warm water circulated to prevent condensation. The microscope was excluded completely from the humid atmosphere. The platform of the microscope revolved around a vertical spindle in the manner of a turntable. Small button magnets were let into it and the eggs placed in an iron-bottomed dish, which adhered firmly to the turntable.

The apparatus was focused by a simple device operated with the feet, capable of raising or lowering the platform of the microscope. The vertical spindle of the turntable passed through the bench and rested on the end of a screw (point upwards). This screw was connected by a flexible cable to a reversible d.c. motor which was controlled with two micro-switches on the floor. The motor had a brake so that, when the current was turned off, it would stop dead without overrunning. With this set-up it was a great advantage to have both hands free at all times.

The observations reported here were based on experiments on White Leghorns. But investigations with other breeds and with ducks were also undertaken. The chick embryos were incubated for 30 hours prior to experimentation.

RESULTS

Observations in the operating incubator

Normal development, for at least 12 hours, would proceed in spite of a very large opening in the vitelline membrane, i.e. one exposing the whole area pellucida. In spite of the high humidity, surface drying still occurred, and evaporation was not, therefore, the causal factor. Clearly, one could not rewet by adding drops of saline or albumen, because in such a long-term procedure topping up with its consequent herniation would be certain to occur sooner or later as one would be adding to the total fluid content of the egg. Instead, the egg was twisted by hand clockwise and anticlockwise on its turntable; when

this was done the yolk, owing to its inertia, was seen to be in more or less constant movement in relation to the shell membrane. This constant movement of the vitelline membrane in relation to the inner surface of the shell membrane wetted it in a manner similar to the action of the eyelid in keeping the cornea moist. Furthermore, it was noticeable, as the egg was twisted first one way and then the other, that the relationship of the blastoderm and vitelline membrane also changed constantly, an additional factor militating against adhesion formation. These observations showed that, so long as the egg was kept in motion in the manner described, the exposed surface of the vitelline membrane remained wet and normal development of the embryo proceeded.

Results with turntable incubator

The next step in the inquiry was to place in the normal incubator a series of mechanically operated horizontal turntables (one for each egg) which rotated clockwise and anticlockwise around a vertical axis through an angle of about 90°, at a speed which could be varied from about 15 cycles per minute downwards. The aim was to reproduce mechanically those movements previously carried out by hand. Windows were cut in the shell and shell membrane in the usual way, and an opening of not less than 0·75 mm. was made in the vitelline membrane. Once the experimental procedure had been completed, the egg was not topped up, the shell window was refixed, and the egg placed on a turntable in the incubator so that the window exactly overlay the centre of the turntable. A speed of 8–9 cycles per minute was found to be the most satisfactory. Of 18 such preliminary experiments, 18 embryos reached stage 19 (Hamburger & Hamilton, 1951), i.e. the stage by which the amnion is completed. Of these embryos, 14 reached hatching. Of a control series of 36 embryos which had been placed in the same incubator but not on the turntables, only 2 reached stage 19, but even these were abnormal in that the area vasculosa was inadequately developed and the amnion was defective; neither survived the 7th day. The turntable incubator was used for a total incubation time of 72 hours, when it was found that the blastoderm usually came to the top with a facility which exceeded that of normal eggs at the same stage, when the eggs were rolled.

Experiments of various kinds concerned with head morphogenesis, all involving major operative procedures, have been performed with this method on embryos ranging from 4 to 12 somites. In all, 120 have been undertaken so far. Most of these experiments were terminated after 8–10 days, but many proceeded for much longer and some to hatching. Of these 120 experiments, 31 were unsuccessful, resulting either in death or in abnormality of the embryo or its membranes. It is very remarkable, however, that only 3 of these 120 embryos failed to reach stage 19. There is no doubt that this technique of incubation has provided one solution of the drying problem.

These results suggest that by this method, given adequate preliminary experience, it is possible to carry out operations during the early somite stages

with the expectation of a success rate comparable to that achieved when the experiments are performed very much later—e.g. for intracoelomic grafting.

Dangers of the method

The speed of rotation on the turntable is limited by the size of the opening in the vitelline membrane. If no opening is made here normal development will occur with speeds up to 15 cycles per minute. On the other hand, if a large opening is made in the vitelline membrane the speed should be as slow as possible, but it must be sufficient to maintain constant movement between the yolk membrane and the shell.

It is very important that the window in the shell membrane should be cut exactly in the right place directly over the embryo. If this has not been done, and the egg has to be tilted to bring the embryo to the central position, it is the author's practice to discard that egg and start again. If the blastoderm becomes eccentric after the experiment has been performed, then it is worth incubating, but a higher failure rate occurs. In fact, of the 31 failures reported above, 24 were described at the time of operation as 'very eccentric' and were therefore given a bad prognosis. But all others similarly described appeared to develop normally. In the remaining seven unsuccessful cases the cause of abnormality was not established.

The relation of the blastoderm to the shell membrane and the way it varies in its behaviour with differently shaped eggs are factors which must be borne in mind when selecting the eggs for experimentation. Eggs in which the blastoderm is not quite at the highest point of the shell, or is too near the blunt end of the egg, should be avoided. The object is to use eggs in which the blastoderm will remain central in position when the egg is rotating.

The only peculiarity which has been noticed is that the head and neck of the embryo begin to fall away from the surface level of the blastoderm into the yolk on the 4th instead of on the 5th day. But this often occurs after intracoelomic grafting and is presumably due to the presence of the air space and not a result of the use of a turntable.

Definitive method

1. Candle the egg and cut window in shell over the blastoderm *c.* 0·5–0·75 cm. square.
2. Place the egg in the operating incubator at temperature of 99·5° F. and nearly 100 per cent. humidity.
3. Candle the egg again and cut window in shell membrane exactly over the embryo.
4. Make opening in vitelline membrane as small as reasonable for experiment required. The position of the opening—over the embryo or to the side—is not important; sticking to the edge of the opening (Silver, 1959) will not occur as long as the vitelline membrane remains wet.

5. Twist the egg on the turntable under the microscope at frequent intervals during the experiment and, finally, as an extra precaution, rewet the whole of the exposed surface, not merely the embryo, with a glass rod or forceps by drawing the peripheral albumen towards the centre, so that it forms an unbroken film. It is now safe to replace and seal the shell window with wax. (The use of adhesive tape or wax paper should in my opinion be avoided because, whenever the incubator door is opened, dew immediately appears on the undersurface and this moisture must come from the suprablastodermal fluid.)
6. Replace the egg in the incubator on its own mechanical turntable (90° turn, alternately clockwise and anticlockwise, 8 cycles per minute) with the window uppermost and immediately over the central vertical spindle of the table. In theory the centrifugal forces acting on the yolk should then balance each other. Each egg should be left on its turntable until a total incubation time of at least 72 hours has elapsed. By this time the amnion is complete and the circulation well established and anti-drying measures are no longer required.
7. It is thought to be inadvisable to roll the eggs during the subsequent weeks of incubation. It is easy to roll 180° immediately after removing the egg from the turntable, but later the albumen, as it becomes viscid, will stick to the window. Once this has happened further attempts to roll the egg will only produce distortion of its contents.

SUMMARY

A method has been described for experimenting on the early chick embryo. By its use it is possible to make an opening in the vitelline membrane large enough for the easy performance of any major operative procedure. The air space over the blastoderm is not topped up. The egg is incubated on a horizontal turntable, rotating around a vertical axis, at about 8 cycles per minute through an angle of about 90°, until the embryo has reached the end of the 3rd day of development. Successful results, using embryos of 4–12 somites, have been achieved in 74 per cent. of 120 major experimental procedures. The rationale of the method has been explained.

RÉSUMÉ

Problèmes spéciaux à envisager pour opérer in ovo *le jeune embryon de poulet, et une solution*

Une méthode est décrite pour opérer le jeune embryon de poulet. Par son emploi, il est possible de faire dans la membrane vitelline une ouverture assez grande pour réaliser facilement n'importe quelle opération importante.

On referme la fenêtre de la coquille, sans emplir de liquide l'espace, contenant de l'air, situé au-dessus de l'embryon.

L'œuf est incubé sur une table horizontale tournant environ 8 fois par minute autour d'un axe vertical et d'un angle d'environ 90 degrés, et cela jusqu'à ce que la fin du troisième jour de développement ait été atteinte. Des opérations graves ont été ainsi réalisées avec succès sur des embryons de 4 à 12 somites dans 74 pour cent des essais effectués. La justification rationnelle de la méthode a été donnée.

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REFERENCES

GRABOWSKI, C. T. (1956). The effects of excision of Hensen's node on the early development of the chick embryo. *J. exp. Zool.* **133,** 301–44.

HAMBURGER, V., & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88,** 49–92.

NEW, D. A. T. (1955). A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. exp. Morph.* **3,** 326–31.

—— (1956). The formation of sub-blastodermic fluid in hen's eggs. *J. Embryol. exp. Morph.* **4,** 221–7.

SILVER, P. H. S. (1957). *In ovo* technique for operating on the early chick embryo. *J. Anat. Lond.* **91,** 605–6.

—— (1959). Embryonic growth relative to the vitelline membrane, and its role in determining the level of post-operative abnormalities, as measured in the chick. *J. Embryol. exp. Morph.* **7,** 564–71.

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Néoblastes et limitation du pouvoir de régénération céphalique chez la planaire *Dendrocoelum lacteum*

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AVEC DEUX PLANCHES

INTRODUCTION

Le pouvoir de régénération des planaires d'eau douce est très variable selon les espèces considérées. Certaines de celles-ci, telle *Euplanaria* (*Dugesia*) *lugubris* O. Schm., régénèrent toujours leur partie céphalique à la suite d'une section transversale, quel que soit le niveau de l'amputation. Chez quelques grandes espèces, comme *Dendrocoelum lacteum* (O.F.M.), la partie postérieure du corps ne reconstitue la région céphalique que si la section a été faite en avant de la limite antérieure du pharynx (fig. 2). De nombreuses hypothèses ont tenté d'expliquer cette limitation du pouvoir régénérateur, hypothèses qu'il est possible de grouper ainsi: influence de la topographie et de la structure nerveuse (Lillie, 1900; Bardeen, 1902; Török, 1958); rôle de facteurs métaboliques ou physico-chimiques (Child, 1911; Brøndsted, 1955; Lender, 1956); et répartition des cellules de régénération (Curtis & Schulze, 1924; Isely, 1925). Certains de ces auteurs pensent d'ailleurs que le pouvoir de régénération est le résultat de plusieurs composantes; par exemple, densité des néoblastes et différences biochimiques.

Dans cette note, il ne sera abordé qu'un point précis de ce problème: existe-t-il des néoblastes (cellules de régénération) dans la région postérieure de *Dendrocoelum lacteum* et, si oui, sont-ils capables d'être activés par une section, de se grouper en un blastème de régénération, et de se différencier pour reconstituer un véritable régénérat céphalique?

L'histologie seule ne peut répondre, même à la première question (répartition des néoblastes), car le dénombrement des cellules de régénération au repos est sujet à bien des erreurs. C'est donc par la voie expérimentale que le problème a été abordé, en se basant sur les résultats obtenus chez *D. lugubris* par Wolff & Dubois (1947), à l'aide d'une technique d'irradiations régionales par les rayons X, dont voici l'essentiel: une région irradiée est rapidement privée de ses cellules

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de régénération, très sensibles aux radio-lésions, surtout lors de leur division à leur arrivée dans la zone cicatricielle. Ces cellules irradiées sont remplacées par leurs semblables saines qui migrent (en deux à plusieurs semaines suivant la distance à parcourir), réparent les lésions de la région irradiée, et reconstituent la région perdue par l'amputation.

TECHNIQUES ET TÉMOINS

Techniques

Les *D. lacteum* sont conservées en élevage à la température de 15° C. et nourries une fois par semaine. Les amputations et irradiations sont faites sous anesthésie au chlorétone en solution aqueuse à 0,13 pour cent. Quelques heures après l'amputation par une section transversale au niveau choisi, les vers sont étalonnés par des fils de repère et soumis à l'action d'une seule dose de rayons X, localisés à une région précise grâce à un écran de plomb (fig. 1). De plus amples détails sont exposés dans le travail de F. Dubois (1949).

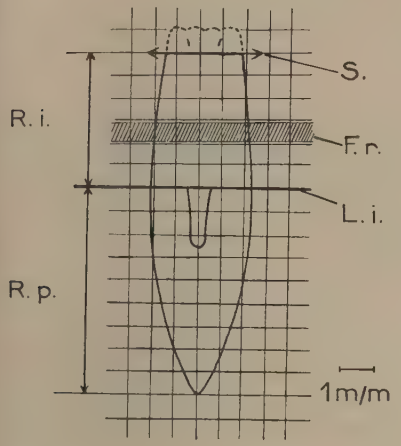

FIG. 1. Principe de l'irradiation régionale. La planaire est recouverte d'un filet muni d'un fil de repère (*F.r.*) perpendiculaire au grand axe du sujet. *L.i.*, limite de l'irradiation; *R.i.*, région irradiée; *R.p.*, région protégée par du plomb; *S.*, section.

Témoins non irradiés (fig. 2)

Les vers sont amputés respectivement derrière les yeux (1ère série), au milieu de la région prépharyngienne (2ème série), et juste en avant du pharynx (3ème série). Chaque série comprend 10 sujets.

1ère série. Le blastème de régénération apparaît le 3ème jour après l'amputation; les taches oculaires deviennent visibles vers le 6ème jour. Vers le 8ème jour, les yeux sont différenciés et la tête est entièrement reconstituée avec ses auricules.

2ème série. Les yeux apparaissent avec un retard de 5 jours par rapport à l'expérience précédente et seulement dans 7 cas.

3ème série. La régénération ne se produit pas.

Ces résultats confirment ceux de Lillie (1900), de Morgan (1904), de Sivickis (1931), et de Brøndsted (1955). Le pouvoir de régénération céphalique chez *D. lacteum* n'existe qu'en avant du pharynx; il diminue d'avant en arrière le long de la région prépharyngienne et devient nul dans la région postérieure du corps à partir de la limite antérieure du pharynx.

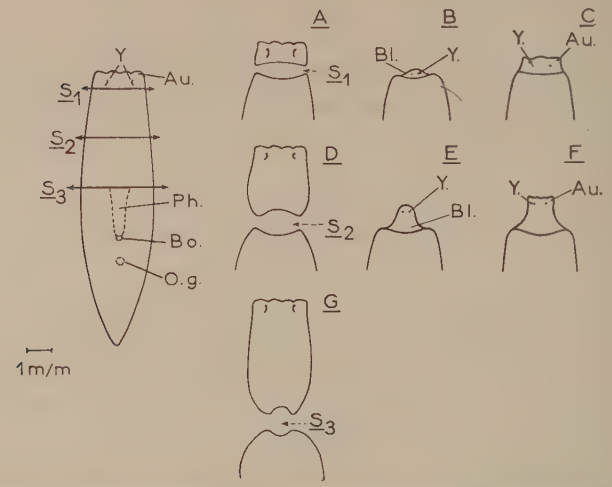

FIG. 2. Régénération céphalique normale. Les trois modes de section sont réunis sur un même schéma. S_1: cas où la section passe juste en arrière des yeux; en *A*, *B*, et *C*, aspects après 6 heures, 6 jours, et 8 jours. S_2: cas intermédiaire; en *D*, *E*, et *F*, aspects après 6 heures, 10 jours, et 15 jours. S_3: cas où la section passe juste en avant de la racine du pharynx; en *G*, aspect après 6 heures, pas de régénération. *Au.*, auricules; *Bl.*, blastème de régénération; *Bo.*, bouche; *O.g.*, orifice génital; *Ph.*, pharynx; *Y.*, yeux.

Témoins irradiés

Dix-sept planaires, dont la tête a été sectionnée en arrière des yeux, sont totalement exposées à l'action des rayons X, dans les conditions qui seront celles des expériences d'irradiations régionales. Un blastème de régénération apparaît à partir du 6ème jour et présente souvent à partir du 8ème jour une ou deux taches oculaires. Puis le blastème involue, les yeux se rapprochent et souvent disparaissent. De toute façon ces yeux ne sont pas fonctionnels. A partir du 20ème jour se manifestent les effets caractéristiques de l'irradiation; le corps est contracté ou tordu, l'épiderme se colore légèrement et prend un aspect granuleux, des boursouflures y apparaissent. La mort survient entre le 21ème et le 31ème jour après l'irradiation.

EXPÉRIENCES D'IRRADIATIONS RÉGIONALES

Trois séries d'expériences sont envisagées suivant l'emplacement de la section et suivant l'ampleur de la surface irradiée (fig. 3).

Section juste en arrière des yeux, irradiation de la moitié antérieure de la région prépharyngienne (fig. 3A, 20 sujets)

Il apparaît un blastème avec des taches oculaires le 8ème jour, mais ce blastème régresse comme dans les cas d'irradiation totale, ceci jusque vers le 20ème jour. A partir de ce moment, la région cicatricielle reprend un aspect sain et un

régénérat oculé normal se développe. Le retard de la régénération des yeux chez ces individus irradiés, par rapport à leurs témoins sains, est de 22 jours en moyenne.

Ces résultats concordent avec ceux de F. Dubois sur *D. lugubris*. Le maigre blastème provisoire, édifié avec des cellules irradiées, disparaît rapidement; le retard apporté à la reconstruction d'un régénérat définitif correspond à la durée de la migration des néoblastes venus de la partie saine.

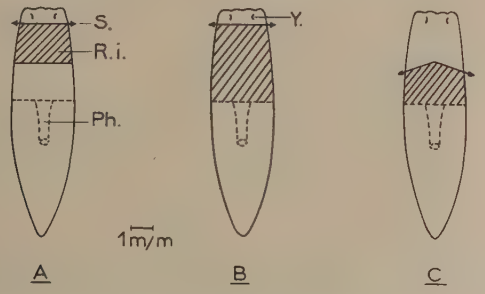

FIG. 3. Amputation suivie d'une irradiation régionale aux rayons X. La région hachurée est irradiée. Schéma des trois séries d'expériences.

Alors que dans le cas de régénération normale, 7 planaires seulement sur 10 ont régénéré à partir des néoblastes de la zone moyenne de la région prépharyngienne (cf. témoins, 2ème série), ici toutes les planaires ont bien régénéré à partir de ces mêmes néoblastes moyens, mais transplantés dans la partie tout à fait antérieure du corps.

Section juste en arrière des yeux, irradiation de toute la région prépharyngienne (fig. 3B, 27 sujets)

Dans ce cas sont détruits tous les néoblastes de la région qui seule est douée du pouvoir de régénérer une tête. Comme dans les cas précédents, un blastème avec deux petites taches oculaires apparaît puis entre très rapidement en état de régression. A partir du 20ème jour, la région irradiée se distingue nettement de la région saine; les effets de l'irradiation y sont analogues à ceux qui marquent les planaires totalement irradiées (fig. 4). Dans les semaines qui suivent, les nécroses s'accentuent au point que des pertes de tissus sont fréquentes, ce qui entraîne parfois la mort de l'animal. Quatre sujets ne se sont jamais régénérés. Mais, et c'est là le résultat essentiel, sur les 20 planaires survivantes, 16 se sont régénérées dans le délai de 8 à 12 semaines après l'irradiation; notons que dans 6 cas les sujets n'ont rien perdu de leurs tissus irradiés (tableau 1). Le régénérat a un aspect sain et se trouve en continuité avec le corps, car les bourrelets et sillons antérieurs de nécrose disparaissent alors; les yeux sont complets et photosensibles. Au cours du développement du blastème, la région irradiée

cesse de se distinguer du reste du corps. Aucun signe de régression ne se manifeste dans les semaines qui suivent la régénération. Celle-ci s'est effectuée grâce à des cellules libres, qui n'ont pu provenir que de la région pharyngienne ou

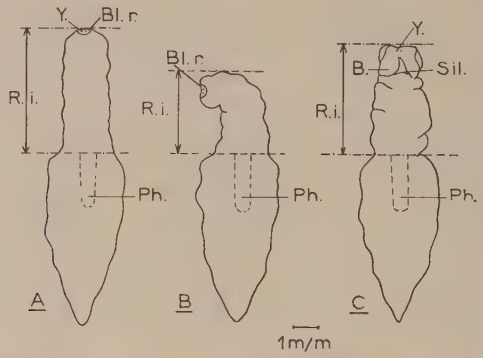

FIG. 4. Aspect des planaires dans le cas où toute la région prépharyngienne est irradiée (cas représenté en fig. 3B). Observations faites sur le vivant, en face dorsale, à partir du 20ème jour après l'irradiation. En *A*, *B*, et *C*, 3 stades de nécroses. *B.*, boursouflure; *Bl. r.*, blastème régressé; *Sil.*, sillon de nécrose.

postpharyngienne. Le long trajet à parcourir explique le grand retard à la régénération et de ce fait les lésions profondes de la région irradiée, privées de cellules réparatrices saines.

TABLEAU 1

Section juste en arrière des yeux, irradiation de toute la région prépharyngienne

Bilan des pertes tissulaires	*Nombre de survivants*	*Taille après section (mm.)*	*Longueur de la région irradiée (mm.)*	*Nombre de cas de régénération céphalique*	*Nombre de cas sans régénération*	*Durée de la régénération (jours)*
Aucune perte	6	9 – 15	3 – 6	6	0	60 – 80
Perte d'environ ½ de la région irradiée	9	9 – 12	4 – 6,5	6	3	54 – 70
Perte d'environ ¾ de la région irradiée	5	12,5 – 14,5	5 – 6,5	4	1	55 – 83
TOTAL	20	—	—	16	4	—
EXTRÊMES	—	—	—	—	—	54 – 83

La régénération a parfois été retardée (avec quelques régénérats aberrants), les bords de la plaie s'étant soudés, soit parce que des pertes de tissus ont provoqué une nouvelle cicatrisation à un niveau postérieur plus riche en fibres

musculaires que la zone céphalique, soit parce que les bords opposés de la section se sont fusionnés par devant le blastème régressé (fig. 5).

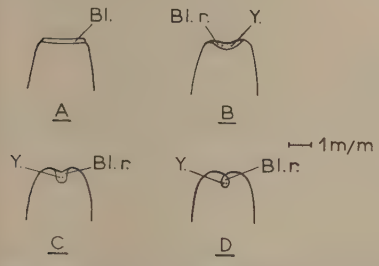

FIG. 5. Soudure des bords latéraux d'un blastème en voie de régression. Irradiation comme en fig. 3B. Observations faites sur le vivant, en face dorsale.

Section en pointe au milieu de la région prépharyngienne, irradiation de toute la région prépharyngienne conservée (fig. 3C, 11 sujets survivants)

Malgré la forme de la section, les bords de la plaie se rapprochent beaucoup et se soudent dans la plupart des cas. Six des onze survivants se sont définitivement régénérés, après une durée de 56 à 90 jours. La proportion des vers incapables de se régénérer est plus forte que dans le cas précédent (c'est-à-dire dans le cas de la section juste derrière les yeux): un peu plus de la moitié au lieu du cinquième, alors que les cellules ont à parcourir une distance plus faible. Cela s'explique aisément par le fait que le pouvoir de régénération céphalique à ce niveau est nettement moins intense qu'au niveau des yeux.

Ainsi apparaissent nettement les deux conditions de la régénération céphalique normale: des néoblastes sains mais aussi une région à haut pouvoir de régénération.

VÉRIFICATIONS HISTOLOGIQUES

Toutes les planaires sont fixées dans le mélange de Bouin et colorées à l'hématoxyline de Heidenhain-éosine. Les coupes ont une épaisseur de 5 μ et sont longitudinales. Tous les sujets sont sectionnés juste derrière les yeux, et les témoins sont sains. Les sujets correspondent à la seconde série expérimentale: irradiation de toute la région prépharyngienne.

Témoins

La cicatrisation s'effectue comme chez les *Euplanaria* (cf. Bartsch, 1923). Au voisinage de la plaie on observe, dès le premier jour qui suit la section, des cellules de régénération piriformes ou fusiformes. Leur nombre s'accroît dans les jours qui suivent, elles forment un blastème dense, très colorable, en arrière duquel d'autres cellules de régénération se pressent en files longitudinales.

A partir du 4ème jour, l'épiderme commence à se reconstituer en avant du blastème. A ce stade, on peut observer au bord antérieur ventral un épiderme mince formé de cellules étirées; ce sont des néoblastes qui ont colonisé la membrane anhyste. Il est possible, en certains endroits, d'observer des néoblastes en train de pénétrer entre les cellules de l'épiderme nouvellement formé. Du côté dorsal, la membrane anhyste subsiste jusqu'au 5ème jour. La différenciation des

néoblastes commence dès leur pénétration dans la membrane anhyste. Sont très visibles notamment les rhabdites, ainsi que les sécrétions éosinophiles qui caractérisent l'organe adhésif au bord ventral antérieur du blastème. Le 6ème jour l'épiderme est complètement reconstitué (planche 1, fig. A), les auricules et les cupules oculaires sont en voie de formation. Les différenciations musculaires et nerveuses ne commencent que le 10ème jour (planche 1, fig. B).

Parmi les cellules qui affluent pour constituer le blastème, certaines sont en mitoses (entre le 4ème et le 6ème jour); mais ces mitoses sont rares; elles ne se situent jamais dans le blastème, mais un peu en arrière de celui-ci.

Sujets d'expériences

Au cours des trois jours qui suivent l'amputation et l'irradiation, la planaire présente une histogenèse normale. C'est à partir du 4ème jour que les différences avec les témoins commencent à être visibles. Les rares néoblastes qui colonisent la membrane anhyste ne se différencient pas. Le long du système nerveux continuent à affluer de nombreuses cellules de régénération, mais certaines d'entre elles possèdent un noyau partiellement détruit; des traînées de matériel chromatique résultent de l'éclatement des noyaux disparus. Contre la pellicule épidermique se forme à partir du 5ème jour un amas important de granulations chromatiques sans trace de noyaux (planche 1, fig. C).

Le blastème lui-même est pauvre en néoblastes, incapable de différenciation; il régresse peu à peu, car l'afflux des néoblastes radio-lésés ne compense plus leur disparition après nécrose *in situ*. Les deux taches oculaires qui sont souvent visibles longtemps dans de tels blastèmes régressés ne sont en réalité que des amas de pigments qui subsistent bien après l'éclatement des cellules pigmentaires.

Vers la 8ème semaine se trouve, au bord tout à fait antérieur des vers, une région vide de cellules, limitée en avant par la membrane anhyste qui présente çà et là une cellule très étirée. L'espace vide correspond à l'ancien blastème dont tous les néoblastes sont morts. Il arrive même que la membrane limitante disparaisse, ainsi que l'épiderme de toute la région irradiée. C'est ainsi que dans la Planche 2, fig. E, on observe le pigment de l'œil en bordure de la région irradiée, le parenchyme directement en contact avec le milieu extérieur. On conçoit que dans ces conditions les désagrégations tissulaires soient fréquentes.

La migration des néoblastes de l'arrière vers l'avant se faisant de façon continue, il arrive un moment (entre la 8ème et la 12ème semaine) où ce sont des cellules saines qui pénètrent dans la région cicatricielle. Pendant leur migration, on ne peut les distinguer des cellules irradiées. Mais leur accumulation en avant du blastème régressé les fait reconnaître en fin de migration. Chez *D. lacteum* toutefois, cette réserve cellulaire qui permet la régénération définitive ne se marque pas extérieurement par le gros bourgeon ventral qui caractérise cette période de la réparation du corps des *E. lugubris* (F. Dubois). Comme chez cette espèce par contre, la colonisation de l'ancien blastème se fait en quelques jours; le blastème définitif est bourré de cellules saines. Sa forme conique, sa

différenciation (un peu lente), ne diffèrent pas de ce qui s'observe chez les témoins (planche 2, fig. F).

La régénération des diverticules digestifs irradiés, à partir de néoblastes sains, s'observe beaucoup mieux chez *D. lacteum* que chez *E. lugubris*.

CONCLUSIONS

L'étude histologique de la régénération céphalique chez la planaire *Dendrocoelum lacteum* souligne la similitude des processus histogénétiques entre cette grande planaire sans pigment, à pouvoir de régénération limité, et d'autres espèces pigmentées, à pouvoir de régénération illimité, telles *Dugesia lugubris*, *Euplanaria polychroa*, ou *Polycelis nigra*, dont l'étude histologique après amputation est bien connue.

Ce sont les cellules libres du parenchyme, dites néoblastes, qui reconstituent toute la partie supprimée. Ces néoblastes arrivent en très grand nombre des régions voisines de la plaie; les premiers se glissent dans la membrane anhyste de cicatrisation pour reformer l'épiderme, les autres s'entassent en un blastème rare en mitoses, qui se différencie rapidement. L'absence de pigment rend plus aisé à suivre que chez les planaires pigmentées les diverses phases des processus régénérateurs. Même en cas de régénération céphalique normale, à partir d'une section tout à fait antérieure, une migration sensible des néoblastes est visible, affectant environ la moitié antérieure de la région prépharyngienne; elle se traduit sur les préparations par un allongement des néoblastes qui deviennent fusiformes ou piriformes.

Les expériences d'irradiation régionale, combinées à des amputations et confirmées par l'étude histologique, mettent en évidence les points suivants.

Il existe des cellules de régénération libres dans la région postérieure à la racine pharyngienne, niveau au delà duquel toute régénération céphalique est impossible. Rappelons qu'une numération ou même une simple observation de ces cellules dans la planaire au repos est difficile, car on confond trop facilement les néoblastes avec d'autres cellules. La méthode expérimentale ne peut d'ailleurs pas permettre de comparer aux différents niveaux la densité en néoblastes, mais elle suffit à affirmer leur présence jusqu'à l'orifice atrial au moins.

Ces néoblastes postérieurs sont capables de migrer vers l'avant, de se grouper en un blastème, de se multiplier, de se différencier en épiderme, intestin, parenchyme. Ce sont donc des cellules qui ne diffèrent en rien des néoblastes de la région prépharyngienne, en particulier leurs potentialités sont les mêmes.

La présence des néoblastes est une condition nécessaire à la régénération, mais non suffisante; cette conclusion rejoint celle des auteurs les plus récents, pour lesquels la limitation du pouvoir de régénération chez les planaires est le résultat de plusieurs composantes (par exemple pour Brøndsted, densité des néoblastes et différences biochimiques). La limitation du pouvoir histogénétique des néoblastes en fonction du niveau de la section est particulièrement mise en

évidence par les expériences d'irradiations régionales. La figure 6 en donne une représentation schématique; comme le nombre de sujets survivants est faible, les résultats n'y sont pas exprimés en pourcentages, mais rapportés à 10 cas dans chaque série; et ils ne visent bien entendu qu'à donner une idée du pouvoir de

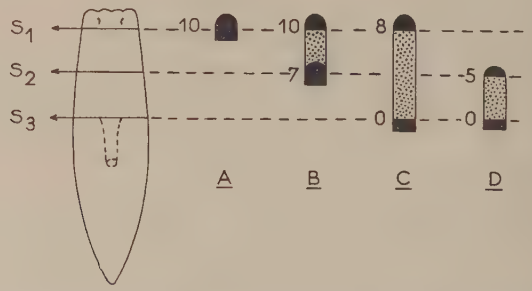

FIG. 6. Schéma du pouvoir histogénétique des néoblastes en fonction du niveau de la section. En pointillé, régions parcourues par des néoblastes en migration. Explication dans le texte.

régénération aux trois niveaux d'amputation. En *A*, une amputation par une section S_1 est régulièrement suivie de régénération. En *B*, les néoblastes qui se différencient 7 fois sur 10 en cas de régénération *in situ* édifient toujours un régénérat quand ils sont transplantés en avant. Enfin, en *C* et *D*, les néoblastes pharyngiens, incapables de révéler leurs potentialités sur place, reconstituent un blastème dans 8 cas sur 10 s'ils sont transplantés très en avant, dans 5 cas s'ils sont amenés à un niveau intermédiaire. En d'autres termes, il n'y a pas de néoblastes 'antérieurs' et 'postérieurs' (ce dont on se doutait depuis longtemps); tous les néoblastes sont identiques et leurs potentialités sont directement fonction de l'endroit du corps où ils sont situés.

En conclusion, le problème de la limitation du pouvoir de régénération chez *Dendrocoelum lacteum* n'est pas lié à la présence ou aux caractères des cellules de régénération. Il se rattache donc à la question bien plus vaste des facteurs inhibiteurs de la régénération, question encore mal connue et qui dépasse largement le cadre de cette note strictement expérimentale.

RÉSUMÉ

1. La régénération de la planaire *Dendrocoelum lacteum* a été vérifiée à trois niveaux différents d'amputation: juste en arrière des yeux, à mi-distance entre le pharynx et les yeux, et juste en avant de la racine pharyngienne. Les planaires se régénèrent toujours dans le premier cas, jamais dans le dernier, environ 7 fois sur 10 dans le cas intermédiaire.

2. Des expériences d'amputation à ces niveaux, combinées à des irradiations régionales par des rayons X, démontrent qu'il existe des cellules de régénération

(néoblastes) dans les régions postérieures à la racine pharyngienne, régions inaptes à la régénération.

3. Ces néoblastes, quand ils ne sont pas irradiés, sont capables de migrer vers l'avant et de réparer les régions irradiées qu'ils traversent; ils reconstituent une région oculée dans un nombre de cas qui dépend directement du niveau prépharyngien de la section.

4. L'histologie confirme les résultats expérimentaux et précise les processus de régression, de nécrose, puis de colonisation des régions irradiées.

SUMMARY

1. The regeneration of the planarian *Dendrocoelum lacteum* has been tested at three different levels of amputation: just behind the eyes, half-way between the eyes and the pharynx, and just in front of the pharyngeal base. The planarians always regenerate in the first case, never in the last case, and about 7 times out of 10 in the intermediate case.

2. Experiments involving amputations and irradiations show that there are regeneration cells (neoblasts) in the regions behind the pharyngeal base, regions which are unable to regenerate.

3. When they are not irradiated, these neoblasts are able to move towards the head and to restore the irradiated regions. They reconstitute a blastema with eyes, the number of cases directly depending on the prepharyngeal level of the section.

4. The histology confirms the experimental results and demonstrates the processes of regression, of necrosis, and then of colonization of the irradiated regions.

TRAVAUX CITÉS

BARDEEN, O. R. (1902). Embryonic and regenerative development in Planarians. *Biol. Bull.* **3**, 262–88.

BARTSCH, O. (1923). Die Histiogenese der Planarienregenerate. *Roux Arch. EntwMech. Organ.* **99**, 187–221.

BRØNDSTED, H. V. (1955). Planarian regeneration. *Biol. Rev.* **30**, 65–126.

CHILD, C. M. (1911). Studies on the axial gradient in *Pl. dorotocephala* as a limiting factor in regulation. *J. exp. Zool.* **10**, 264–320.

CURTIS, W. C., & SCHULZE, L. M. (1924). Formative cells of Planarians. *Anat. Rec.* **29**, 105.

DUBOIS, F. (1949). Contribution à l'étude de la migration des cellules de régénération chez les Planaires. *Bull. Biol. Fr. Belg.* **83**, 213–83.

ISELY, M. F. (1925). The power of regeneration in three genera of Planarians: *Planaria*, *Phagocata*, *Dendrocoelum*. *Anat. Rec.* **31**, 305–6.

LENDER, T. (1956). Analyse des phénomènes d'induction et d'inhibition dans la régénération des Planaires. *Année biol.* **32**, 457–71.

LILLIE, F. R. (1900). Some notes on regeneration and regulation in Planarians. *Amer. Nat.* **34**, 173–7.

MORGAN, T. H. (1904). Notes on regeneration. *Biol. Bull.* **6**, 159–72.

SIVICKIS, P. B. (1931). A quantitative study of regeneration in *D. lacteum*. *Arb. ung. biol. ForschInst.* **4**, 1.

STÉPHAN-DUBOIS, F., & KOLMAYER, S. (1959). La migration et la différenciation des cellules de régénération chez la Planaire *D. lacteum*. *C.R. Soc. Biol. Paris*, **153**, 1856–8.

Török, L. J. (1958). Experimental contributions to the regenerative capacity of *Dugesia lugubris*. *Acta biologica* (*Hungary*), **1**, 79–98.

Wolff, Ét., & Dubois, F. (1947). La migration des cellules de régénération et les facteurs qui la provoquent chez les Triclades. *C.R. Acad. Sci. Paris*, **224**, 1387–8.

EXPLICATION DES PLANCHES

Lettres communes aux figures des planches 1 et 2: *Bl.*, blastème de régénération; *C.r.*, cellules de régénération; *Ep.*, épiderme; *F.a.*, fossette adhésive; *Mb.*, membrane de cicatrisation; *Oe.*, œil; *Par.* parenchyme; *Rh.*, rhabdites; *S.n.*, système nerveux.

Planche 1

Fig. A. Régénérat céphalique normal de 6 jours. Coupe sagittale. Nombreuses cellules de régénération en avant du système nerveux ancien. Épiderme nouveau avec ciliature, rhabdites.

Fig. B. Régénérat céphalique normal de 9 jours. Coupe parallèle au plan sagittal. Le blastème s'est allongé. L'œil commence à se différencier, avec cupule pigmentaire.

Fig. C. Blastème de régénération de 5 jours, après irradiation régionale. Coupe sagittale. Les modalités de l'irradiation par les rayons X sont exposées dans la fig. 3B du texte. Épiderme pauvrement représenté ventralement; dorsalement, la membrane de cicatrisation n'est pas colonisée. Blastème réduit à un amas de cellules de régénération en cours de nécrose. Quelques cellules de régénération se dirigent vers le blastème; leurs radio-lésions se manifesteront lors de leur division, à leur entrée dans le blastème.

Planche 2

Fig. D. Région antérieure sans régénérat, amputée depuis 4 semaines. Vue partielle, coupe sagittale. Irradiation comme en fig. C. Il n'y a presque plus de cellules de régénération dans la zone cicatricielle qui est vacuolisée; la membrane anhyste est étirée à l'extrême.

Fig. E. Région antérieure sans régénérat, amputée depuis 6 semaines. Vue partielle, coupe parallèle au plan sagittal. Irradiation comme en fig. C. Les cellules de régénération saines commencent à se grouper en avant du cordon nerveux. La membrane cicatricielle s'est déchirée, le parenchyme et l'amas de pigment de l'œil régressé sont directement en contact avec l'extérieur.

Fig. F. Régénérat définitif de 10 semaines. Coupe parallèle au plan sagittal. Irradiation comme en fig. C. Nombreuses cellules de régénération. Épiderme cilié, œil bien différencié, fossette adhésive avec cellules éosinophiles.

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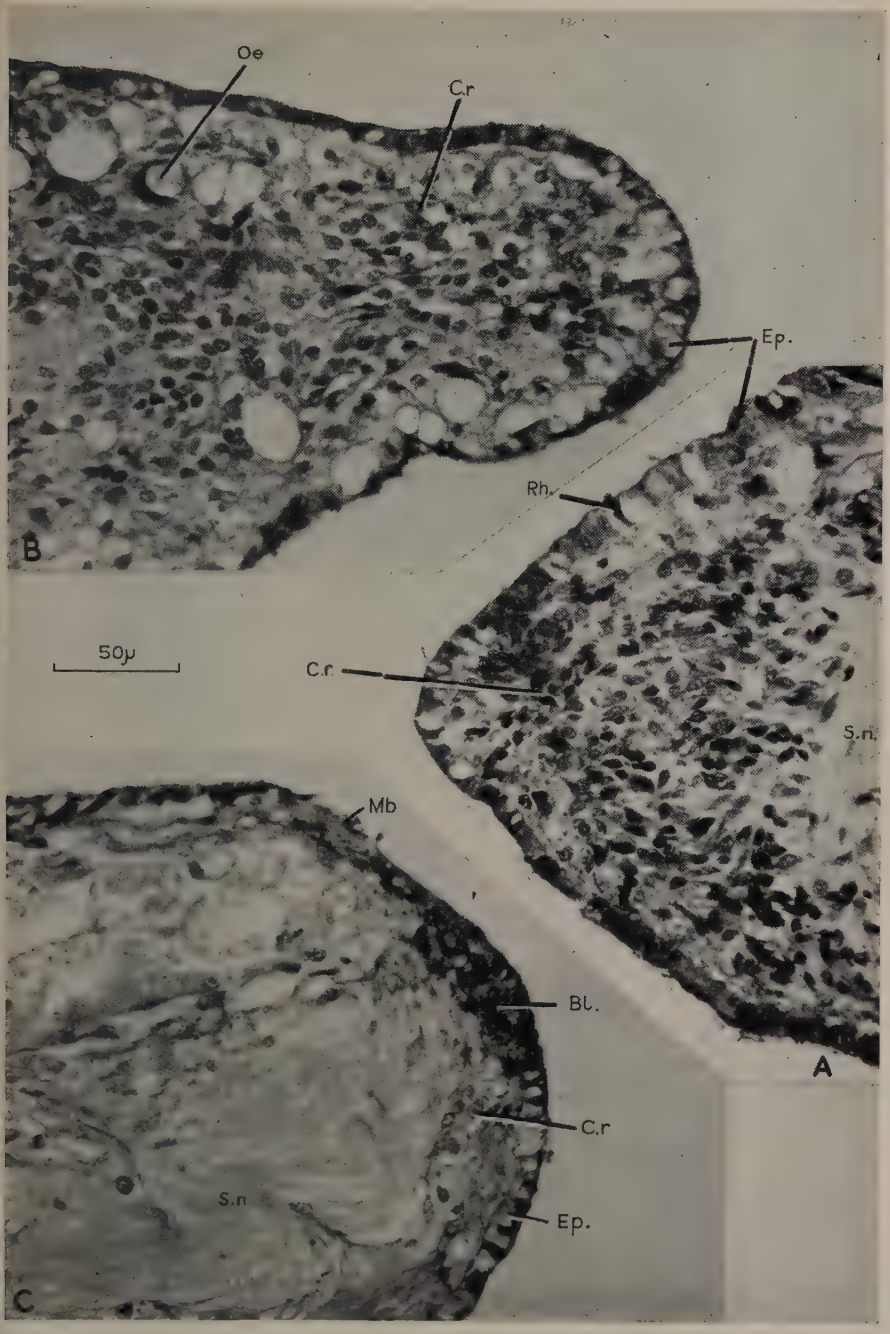

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Planche 1

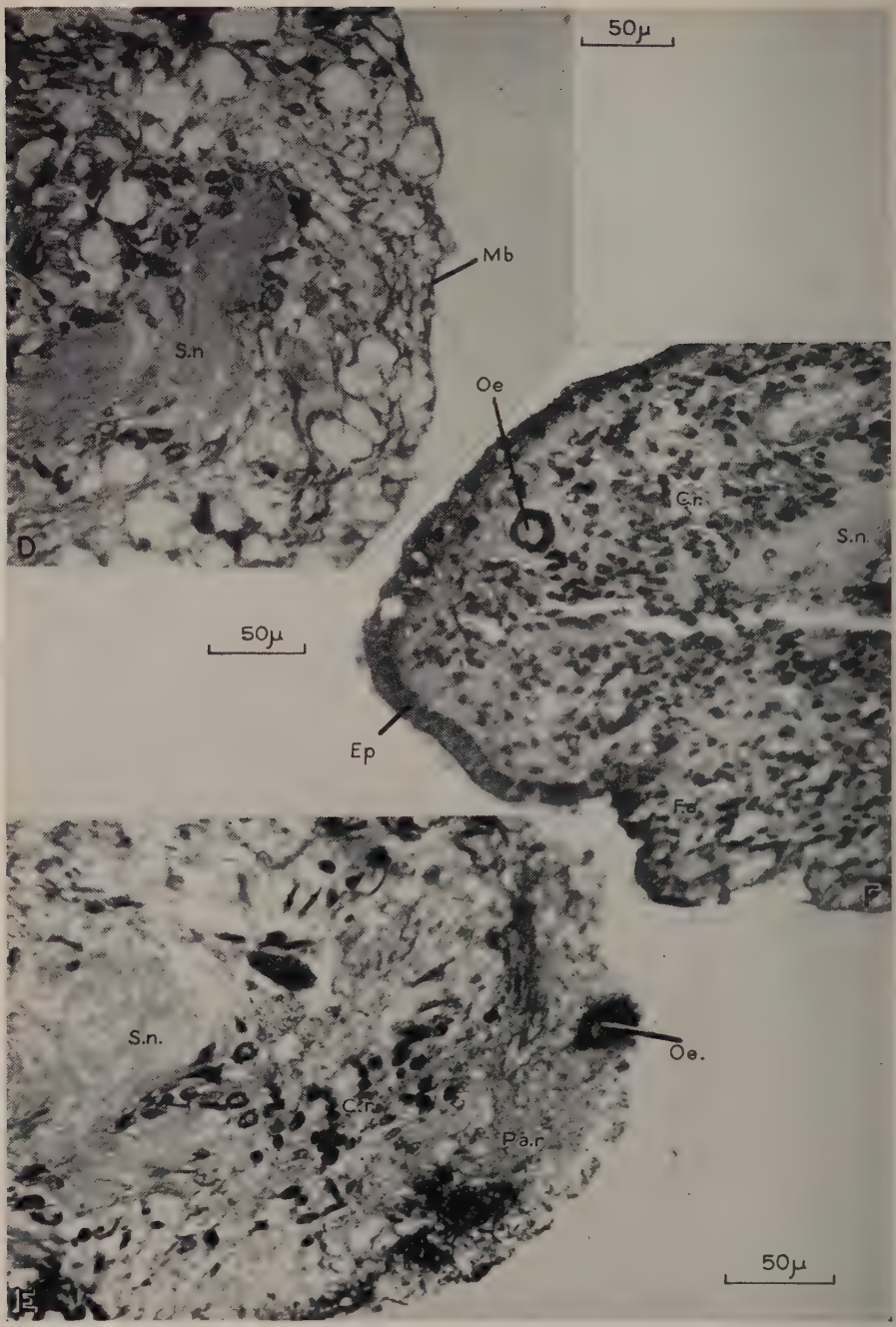

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Planche 2

The Skin Abnormality of 'Ichthyosis', a Mutant of the House Mouse

by R. I. SPEARMAN[1]

From the Dermatology Department, University College Hospital Medical School

WITH ONE PLATE

THE skin defect 'ichthyosis' of the house mouse is caused by a simple recessive gene (symbol *ic*) which arose as a spontaneous mutation. It was first described by Carter & Phillips (1950). 'Ichthyotic' mice have a thin coat of very short wavy hairs together with a scaly skin. There is, however, considerable individual variation in the severity of the defect. In badly affected animals the stratum corneum of the back is shed as large flakes, and the mice may be almost bald; but in less affected mice the skin is not very scaly and there is a complete though abnormal hair cover. The tail skin has a hard smooth appearance and is less flexible than normal. In some older mice a number of depressed rings form along its length, and a portion of the tail undergoes necrosis and drops off distal to one of the constrictions.

Female ichthyotic mice are usually sterile, as are some of the males. In the present investigation fertile *ic*/*ic* males were obtained from Dr. T. C. Carter, and these were outcrossed to normal CBA strain females from Professor H. Grüneberg's laboratory. An ichthyotic stock was maintained by back-crossing +/*ic* females to *ic*/*ic* males. These mice were generally less severely affected than those originally described by Carter & Phillips (1950).

The present paper describes the structure of the abnormal hairs and skin of the ichthyotic mouse, examined by routine and fluorescence microscopy (Jarrett, Bligh, & Hardy, 1956). Measurements of hairs and of the thickness of the tail epidermis are compared with measurements for the normal mouse.

INVESTIGATION

The coat

Hairs plucked from the backs of ichthyotic and normal mice were examined microscopically after mounting in Canada Balsam. Ichthyotic hairs were clearly distinguished from normal mouse hairs. The *awl*, *auchene*, *zigzag*, and *guard* hairs, as described by Dry (1926) for the normal coat, were not easily identified.

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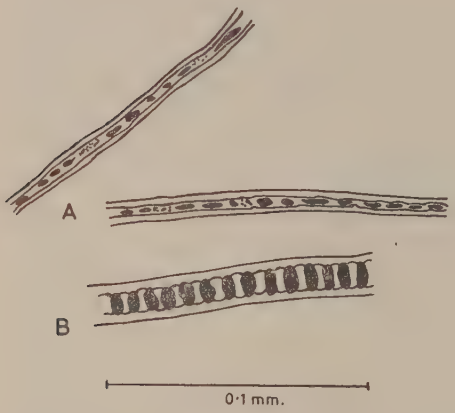
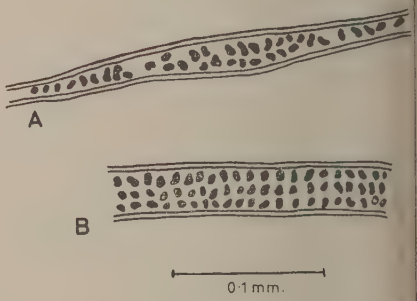
All ichthyotic hairs were wavy, and varied in thickness along their length due to alterations in size of the medulla. The most common type of unilocular hair of these animals (Text-fig. 1A) seems to correspond to the normal zigzag hair (Text-fig. 1B) and has a single row of air spaces; but in the ichthyotic hairs the medulla is compressed and there are no constrictions or angle bends. A smaller proportion of hairs have a multilocular medulla with air spaces varying from 1 to 3 in number, and they are arranged in a haphazard manner (Text-fig. 2A). This type probably corresponds to the normal awl and auchene hairs (Text-fig. 2B). Hairs approximating to normal awls were seen in some slightly affected animals. Long unilocular hairs possibly represent guard hairs. In some places in the medulla there were no melanin granules. There are gradations between the different types of hair, the most affected mice having the most deformed hairs.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. A, ichthyotic mouse unilocular hairs from back showing compressed medulla. Camera lucida drawing. B, Normal mouse unilocular *zigzag* hair from back. Camera lucida drawing.

TEXT-FIG. 2. A, ichthyotic mouse multilocular hair from back, showing variation in width and haphazard arrangement of medullary melanin granules. Camera lucida drawing. B, normal mouse multilocular *awl* hair from back. Camera lucida drawing.

Ichthyotic and normal mouse hairs were fluorochromed with 0·1 per cent. acridine orange and 0·1 per cent. rhodamine B. After dehydration and clearing the hairs were mounted in DePex, and examined by fluorescence microscopy under UV light in the manner described by Jarrett & Spearman (1957) for the mutant 'matted'. The acridine orange did not penetrate the ichthyotic hairs and fluoresce in the medula as in matted mouse hairs. This suggests that the hair cuticle is not defective. Hairs did not show splitting or breakage. The cortical keratin of ichthyotic hair fluoresced salmon pink in places with rhodamine B, unlike normal hair cortex, which was non-fluorescent. This indicates that the hair keratin is abnormal even though splitting is uncommon.

Plucked hairs from 12 ichthyotic and 12 normal mice were examined microscopically. Hair lengths and fibre widths were measured. The results are shown in Table 1, and it is evident that ichthyotic mice have finer and shorter hairs than normal mice.

TABLE 1

Comparison of mean hair measurements from middle of back for 12 ic/ic *and 12 normal CBA mice*

Type of hair	*Mean thickness in μ*	*S.D.*	*Mean length in mm.*	*S.D.*
Normal awl (multilocular fibre)	34·7	±3·0	6·1	±0·52
ic/ic (multilocular fibre)	18·1	±6·1	2·76	±0·56
Normal zigzag (unilocular fibre)	17·6	±2·6	5·3	±0·74
ic/ic (small unilocular fibre)	7·16	±0·8	2·3	±0·32

Dorsal skin from 3 bald ichthyotic and 3 normal mice was fixed in Bouin's fluid and then macerated in dilute acetic acid according to Dry's (1926) method. The panniculus muscle and fat were removed, and counts were made of the hair follicles in a given flat area of skin. No deviation from the normal was detected in follicle density. By reversing the slide the emergent hairs were examined, but again no numerical deviation was seen. The skin of 6 nearly bald adult ichthyotic mice was examined under the low power of a binocular dissecting microscope. Despite the hairless appearance, the skin was seen to be covered with short fine 'lanugo' hairs.

The hairless appearance of ichthyotic mice is, therefore, caused by the dwarfed nature of the hairs.

HISTOLOGY

The epidermis

Skin from the back and tail of 12 ichthyotic and 12 normal mice was fixed in 70 per cent. alcohol (Jarrett & Hardy, 1957) and cut at 7 μ in paraffin wax. Sections were stained in haematoxylin and eosin. Other sections for fluorescence microscopy were stained for 40 minutes in an aqueous mixture of 20 parts 0·02 per cent. congo red and 10 parts 0·1 per cent. titan yellow, washed in water, and then stained for 3 minutes in 0·1 per cent. thioflavine T. The congo red/titan yellow mixture was freshly prepared and the technique is fully described by Jarrett, Spearman, & Hardy (1959).

Normal mouse-tail skin had a granular layer around the hair follicles but not under the tail scales (Plate, fig. A). Ichthyotic mouse-tail skin had a patchy granular layer in both the follicular and scale regions, and the thickened tail epidermis had enlarged cells and many more active nucleoli than normal skin (Plate, fig. B).

Normal mouse stratum corneum in the follicular region of the tail and over the back fluoresced red with the congo red and thioflavine T technique, but the 'hard' keratin of the tail scales fluoresced blue (Jarrett *et al.*, 1959). Ichthyotic mouse follicular tail-skin keratin had the same blue colour fluorescence as the tail scales. The tail stratum corneum was thicker and more flaky than normal, but had no nuclear chromatin remains. The ichthyotic back horny layer fluoresced red by this congo red staining method.

Follicular structure

The structure of the hair follicles on the back of 3 ichthyotic and 3 normal mice was examined. Follicle growth was stimulated by plucking the hairs (Montagna, 1956); the mice were killed 26 days after plucking. Both ichthyotic and normal hair follicles were stimulated to the same phase of anagen development. Ichthyotic follicles did not appear abnormal, and the arrangement of the keratinized inner root-sheath was similar to that found in the normal mouse. Club hairs were seen in the resting areas of ichthyotic skin, as in the normal mouse skin.

Epidermal thickness

Tail epidermal thickness was measured in 12 normal CBA mice and 12 ichthyotic mice. Ten measurements were made for each animal in the mid-scale regions. The width was measured from the basal layer to the bottom of the horny layer in haematoxylin and eosin stained sections. The mean thickness of the normal tail epidermis was 26·4 $\mu \pm$S.D. 2·0, but the thickness of the ichthyotic tail epidermis was 59·8 $\mu \pm$S.D. 15·0. The variation in thickness of the mutant epidermis reflects the different degrees of severity of the defect.

Histochemistry

Sections of skin from 3 ichthyotic and 3 normal mice were examined by a modified Baker's acid haematin method for phospholipids (Jarrett *et al.*, 1959). Although some lipids and absorbed sebum were removed during processing, normal tail skin showed a reaction in the scale keratin layer, but not around the follicles. It is thought that this is due to protein-bound phospholipids. Ichthyotic mouse-tail stratum corneum had a uniform reaction for phospholipids in both follicular and scale regions.

In normal alcohol fixed tissue only nuclear proteins fluoresce with thioflavine T. The epidermal cytoplasmic RNA was examined in sections of tail skin stained with 0·1 per cent. thioflavine T after removal of the DNA with the enzyme DNAase. Ichthyotic tail epidermis showed a stronger yellow colour fluorescence than normal mouse skin, indicating a higher content of RNA. This technique has been described by Jarrett (1958) and Jarrett *et al.* (1959). The findings of a high cytoplasmic RNA content, together with active nucleoli in ichthyotic epidermis, suggests that protein synthesis is increased (Davidson, 1960).

Normal epidermal keratin fluoresces blue with thioflavine T, but after oxidation of sections in 3 per cent. peracetic acid for 25 minutes it fluoresces yellow with this fluorochrome. Peracetic acid has little effect on the unkeratinized epidermis, and it is thought that this acid specifically oxidizes the cystine of the keratin (Alexander & Hudson, 1954) and renders it stainable (Fraser & Rogers, 1955; Jarrett *et al.*, 1959). Therefore, keratins having a high cystine content (as in hair) fluoresce a brighter yellow than those with a lower cystine content. Normal mouse-tail skin fluoresced a deeper yellow in the scales than in the keratin around the follicles. Ichthyotic tail keratin, however, fluoresced strongly in both regions after oxidation. This suggests that the keratin around the tail-hair follicles of the ichthyotic mouse has an abnormally high cystine content. Sections of skin were also examined for protein-bound sulphydryl groups by the method of Barrnett & Seligman (1952). Normal tail-epidermal keratin showed a reaction only in the scales, but the mutant tail had a uniform reaction for bound sulphydryl groups in both the scale and perifollicular horny layers.

The tail constrictions

Tail-skin containing deep depressed rings was examined histologically in 3 mice. Sections were stained in haematoxylin and eosin, and by the congo red/thioflavine T technique. The epidermis appeared to be drawn down against the panniculus carnosus muscle and the dermal tissue was missing in this region (Plate, fig. C). No alteration was detected in the epidermal cells of the depressed ring, either by routine staining or by fluorescence microscopy. Constrictions were, therefore, not due to overlying keratin pressing down on the epidermis, as had been suggested as a possible cause (Carter & Phillips, 1950). It has been shown by Jarrett *et al.* (1959) that compressed epidermal cells undergo a form of keratinization, and that even slight pressure causes the liberation of nucleic acids from the nucleus into the cytoplasm. It is likely that local atrophy of the dermis produces ringing. Pressure on blood-vessels in the affected region would then lead to necrosis and shedding of the distal portion of the tail. Ichthyotic mice were found especially liable to impetiginous skin infections, and the epidermis at the constrictions often became secondarily infected.

COMPARISON OF THE TAIL-SKIN OF MOUSE MUTANT 'ICHTHYOSIS' WITH HUMAN ICHTHYOSIS AND PSORIASIS

Sections of ichthyotic mouse-skin were compared with sections of skin from subjects suffering from ichthyosis vulgaris and psoriasis. These are human disorders with abnormal keratinization, thought to have a genetic basis. Both defects, as in ichthyotic mouse-tail skin, have an abnormally high content of sulphydryl groups and protein-bound phospholipids in the stratum corneum (Jarrett *et al.*, 1959). Human ichthyosis has a thin atrophic epidermis, but psoriasis, like the mutant mouse-tail, has an abnormally thick epidermis containing numerous active nucleoli: stainable nuclear remains are absent from

the horny layer in human ichthyosis and in the mutant mouse, but in psoriasis there is a parakeratotic horny layer with altered nuclei.

In human ichthyosis and in the ichthyotic mouse there is a thin poorly developed granular layer, but this is entirely absent in true psoriasis. Psoriasis has an abnormally increased dermal vascularity, which produces a clinical reddening of the skin, but no such vascular change is seen either in mouse or human ichthyosis. In neither of the human disorders is there any alteration in hair structure.

In the human disorders, similar histochemical changes in the stratum corneum can be produced either by an atrophic or by a physiologically active epidermis: the mouse mutant has features resembling both human ichthyosis and psoriasis. The epidermal horny layer resembles human ichthyosis in that there are no nuclear remanents, but the increased epidermal activity is more allied to psoriasis.

DISCUSSION

The thinness of the ichthyotic mouse-coat is due to the abnormal nature of the hairs. There does not appear to be a suppression of follicle growth as occurs in the mutants *crinkled* (Falconer *et al.*, 1951) and *ragged* (Slee, 1957). Hairs do not fragment as in *naked* (David, 1932, 1934) or *matted* (Searle & Spearman, 1957). Widespread depilation of whole hairs as in *hairless* (Fraser, 1946) was not seen.

Normal mouse-tail skin has a flexible 'soft' stratum corneum around the hair follicles with a more rigid 'hard' keratin in the tail scales (Jarrett *et al.*, 1959). This arrangement allows freedom of movement of the tail. Ichthyotic tail skin has a rigid type of horny layer in both follicular and scale regions, and this would interfere with tail movements.

The rigid type of horny layer in ichthyotic tail skin is formed from a patchy granular layer. This is unusual because in normal skin a granular layer is associated with the formation of flexible keratinized cells. Ichthyotic epidermis is active and it is possible that the rate of cell movement from basal to horny layer is greater than normal. If the rate of cell turnover is increased, there may be too little time for enzyme systems thought to be concerned in flexible keratin formation to act before the epidermal cells become cornified (Jarrett *et al.*, 1959). In these circumstances one might expect the formation of an abnormal keratin layer. It is interesting that no keratin abnormality of the back skin was detected by fluorescence microscopy. It is possible that ichthyotic mice with more severely affected backs than those examined might show an altered keratinization.

The fact that both hairs and epidermis are affected indicates that the primary abnormality lies in the skin, as was suggested by Grüneberg (1952). The dermis is thought to control the pattern of epidermal development, and epidermal cells in tissue culture become organized into epidermis only when dermal cells are

present (Montagna, 1956). Possibly there is an induction of abnormal epidermis by the dermis in the ichthyotic mouse-tail. The peculiar ringing which takes place in the ichthyotic mouse-tail skin appears to be due to atrophic changes in the dermal connective tissue. The immediate cause of these changes is not clear.

In the human race an abnormality known as Ainhum occurs among African negroes. In this defect there is a similar formation of depressed rings around the digits (Sutton, 1956; Clarke, 1959). The cause of this disorder is uncertain but it has been suggested that it has a genetic basis. Keratoma mutilans referred to by Gates (1946) as a dominant gene defect in man may be a form of Ainhum. The epidermis in Ainhum is reported to be drawn down in a furrow against the underlying bone and spontaneous amputation later occurs as in the ichthyotic mouse-tail.

In the condition known as ring tail (Worden & Lane-Petter, 1957), which occurs in new-born rats, depressed rings form along the tail and the part distal to a constriction eventually drops off as in the ichthyotic mouse and in Ainhum. The pathology of this defect does not seem to have been investigated and the cause is uncertain.

SUMMARY

1. Hair and skin from the mouse mutant 'ichthyosis' have been studied by routine and by fluorescence microscopy. Ichthyotic mouse-hairs from the back were shorter and finer than normal. All hairs are wavy, and have an unevenly compressed medulla with an irregular distribution of melanin granules. Hairs do not fragment. No deviation from normal was found in hair follicle counts even in bald areas, but the dwarfed hairs are unable to form an adequate hair cover.

2. The tail epidermis was thicker and more active than normal, and in contrast to normal tail skin there was a patchy granular layer in the scale regions.

3. The stratum corneum around the hair follicles of the tail was histochemically similar to the hard keratinized scales of the normal mouse. There was also hyperkeratosis of both the follicular and tail scale regions. No abnormality was detected in the epidermis of the back by the methods employed.

4. Depressed rings form along the tail and the epidermis is drawn down against the panniculus muscle. This is probably due to dermal atrophy. The defect simulates a syndrome known as Ainhum, which occurs in African negroes.

5. The keratinization defects are compared in mouse ichthyosis and in the human disorders ichthyosis vulgaris and psoriasis.

RÉSUMÉ

Les Anomalies de la peau chez le mutant 'ichthyosis' de la Souris domestique

1. Le poil et la peau de la Souris portant la mutation 'ichthyosis' ont été étudiés au microscope ordinaire et au microscope à fluorescence. Les poils du

dos des Souris ichthyosis sont plus courts et plus fins que normalement. Tous les poils sont ondulés. Ils présentent une moelle inégalement comprimée et une distribution irrégulière des grains de mélanine. Les poils ne se fragmentent pas. Le nombre des follicules pileux n'est pas modifié par rapport au nombre normal, même dans les régions chauves, mais les poils nains sont incapables de former une fourrure adéquate.

2. L'épiderme de la queue est plus épais et plus actif que l'épiderme normal. Il existe dans les écailles des mutants une couche granuleuse morcelée, alors que celle-ci est absente chez la Souris normale.

3. Histochimiquement, le stratum corneum autour des follicules pileux est semblable à celui des écailles de kératine dure de la peau normale. On ne constate, avec les méthodes employées, aucune anomalie de l'épiderme dorsal.

4. La queue des mutants porte une série de constrictions annulaires, au niveau desquelles l'épiderme est appliqué contre le muscle du pannicle. Cela est probablement dû à une atrophie du derme. Cette malformation mime l'ainhum des Nègres africains.

5. Les anomalies de la kératinisation chez la Souris mutante ichthyosis sont comparées aux affections humaines ichthyosis vulgaris et psoriasis.

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REFERENCES

Alexander, P., & Hudson, R. T. (1954). *Wool, its Chemistry and Physics*, p. 265. London: Chapman & Hall.

Barrnett, R. J., & Seligman, A. M. (1952). Histochemical demonstration of protein-bound sulphydryl groups. *Science*, **116**, 323–7.

Carter, T. C., & Phillips, R. S. (1950). Ichthyosis, a new recessive mutant in the house mouse. *J. Hered.* **41**, 297–300.

Clarke, G. H. V. (1959). *Skin Diseases in the African*, p. 46. London: H. K. Lewis.

David, L. T. (1932). The external expression and comparative dermal histology of hereditary hairlessness in mammals. *Z. Zellforsch.* **14**, 616–719.

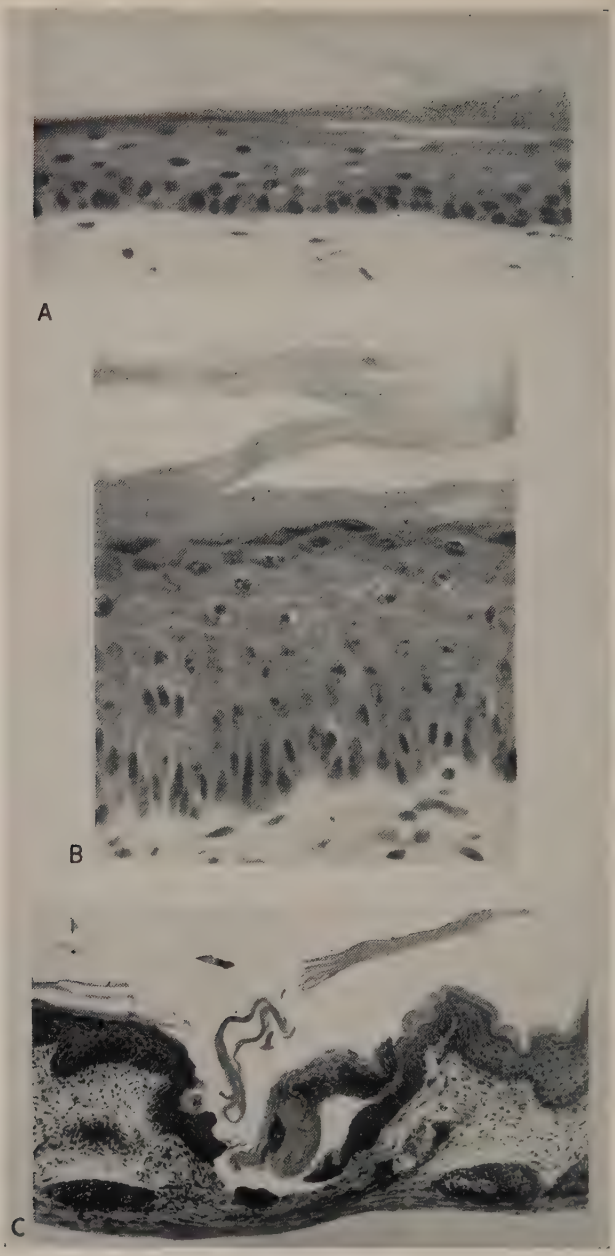
—— (1934). Studies on the expression of genetic hairlessness in the house mouse. *J. exp. Zool.* **68**, 501–18.

Davidson, J. N. (1960). *The Biochemistry of the Nucleic Acids*, p. 231, 4th ed. London: Methuen.

Dry, F. W. (1926). The coat of the mouse. *J. Genet.* **16**, 287–340.

Falconer, D. S., Fraser, A. S., & King, J. W. B. (1951). The genetics and development of *crinkled*, a new mutant in the house mouse. *J. Genet.* **50**, 324–44.

Fraser, F. C. (1946). The expression and interaction of hereditary factors producing hypotrichosis in the house mouse: histology and experimental results. *Canad. J. Res.* (D), **24**, 10–25.



R. I. SPEARMAN

Fraser, R. D. B., & Rogers, G. E. (1955). The bilateral structure of wool cortex. *Proc. Inst. Wool Text. Res. Conf.* (*Australia*), pp. 151–5.

Gates, R. R. (1946). *Human Genetics*, vol. 1, p. 336. New York: MacMillan.

Grüneberg, H. (1952). *The Genetics of the Mouse*, p. 102, 2nd ed. The Hague: Martinus Nijhoff.

Jarrett, A. (1958). The Chemistry of inner root sheath and hair keratins. *Brit. J. Derm.* **70,** 271–83.

—— & Hardy, J. A. (1957). The value of alcohol for fixation of skin. *Stain Tech.* **32,** 225–33.

—— & Spearman, R. I. (1957). The keratin defect and hair cycle of a new mutant (matted) in the house mouse. *J. Embryol. exp. Morph.* **5,** 103–10.

——, Spearman, R. I., & Hardy, J. A. (1959). The histochemistry of keratinization. *Brit. J. Derm.* **71,** 277–95.

——, Bligh, A., & Hardy, J. A. (1956). Fluorescent microscopy of the human skin. *Brit. J. Derm.* **68,** 111–19.

Montagna, W. (1956). *The Structure and Function of Skin*, pp. 240, 332. New York: Academic Press.

Searle, A. G., & Spearman, R. I. (1957). 'Matted', a new hair mutant in the house mouse: genetics and morphology. *J. Embryol. exp. Morph.* **5,** 93–102.

Slee, J. (1957). The morphology and development of 'ragged'—a mutant affecting the skin and hair of the house mouse. I. Adult morphology. *J. Genet.* **55,** 100–21.

Sutton, R. L. (1956). *Diseases of the Skin*, p. 1027, 11th ed. London: Kempton.

Worden, A. N., & Lane-Petter, W. (1957). *UFAW Handbook on the Care and Management of Laboratory Animals*, p. 366, 2nd ed. London: U.F.A.W.

EXPLANATION OF PLATE

Fig. A. Normal mouse-tail scale epidermis stained with haematoxylin and eosin. No granular layer is present. Sagittal section. ×600.

Fig. B. Ichthyotic mouse-tail scale, showing the thickened epidermis containing enlarged cells with active nucleoli. In contrast to the normal tail scale a granular layer is present under the hyperkeratotic stratum corneum. H. & E. stained sagittal section. ×600.

Fig. C. Ichthyotic mouse-tail skin, showing a sagittal section through a tail constriction. The dermis is atrophied beneath the groove formed by the epidermis. Secondary impetiginous infection has occurred and inflammatory cells are seen in the stratum corneum. H. & E. stained section. ×140.

The Effect of Benzimidazole on the Differentiation of Ectodermal Explants from the Gastrulae of *Xenopus laevis*

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WITH ONE PLATE

INTRODUCTION

THE treatment of amphibian and chick embryos during the early stages of development with small concentrations of benzimidazole, and some of its derivatives, results in the formation of abnormal embryos (Liedke, Engleman, & Graff, 1954; Waddington, Feldman, & Perry, 1955*a*; Billett & Perry, 1957 *a*, *b*). The gross effects produced by these substances are of a fairly general kind. Abnormal gastrulation, decomposition of neurulae, and microcephaly are produced in Amphibia. Rather less well-defined abnormalities, involving the head, neural tube, and somites, are seen in the chick.

The way in which these abnormalities are produced is not known. The idea that benzimidazole acts simply as a purine anti-metabolite is not well founded (Slonimski, 1954). The work of Tamm and his colleagues (Tamm, Folkers, Shunk, & Horsfall, 1953; Tamm, 1958) has shown that benzimidazole and certain of its derivatives inhibit the growth of some viruses. The nature of some of these derivatives, and some recent biochemical observations (Allfrey *et al.*, 1957; Tamm, 1957), suggest strongly that the compounds interfere in some way with ribonucleic acid metabolism. Although there is no direct evidence to confirm the view, it is conceivable that the abnormalities produced in embryos by benzimidazole and its derivatives are caused primarily by disturbances in ribonucleic acid metabolism.

Examination of newt embryos treated with benzimidazole at the neurula stage shows that cell degeneration is particularly evident in the neural tissue and is also seen to some extent in the newly formed mesenchyme (Waddington *et al.*, 1955*a*). A similar pattern of damage was seen in *Xenopus* and chick embryos treated with benzimidazole and its alkyl derivatives (Billett & Perry, 1957 *a*, *b*).

Although the localized cell damage seen in the intact embryos may be the

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result of the direct effect of benzimidazole on the cells, this is by no means certain. The compound may act by reducing the metabolic activity of all the cells in the embryo, and disintegration might then start in localized areas of cells most sensitive to a general toxic effect. Dividing or differentiating cells might come within this category. Alternatively, abnormalities of gastrulation might occur and neurulation could then be upset by the disturbance of normal inductive relationships. For instance, an embryo having undergone a partial exogastrulation might develop a retarded head and show extensive cell damage in the forebrain region.

Difficulties of interpretation can be overcome to a certain extent by using isolated pieces of embryonic ectoderm capable of a limited amount of differentiation. Such explants, readily obtained from amphibian gastrulae, are relatively simple systems in which a small number of well-defined cell types arise during the limited life of the explants. Compared with whole embryos the explants are small and their external surface is uniform. Under these conditions a fairly rapid and even penetration of benzimidazole is to be expected.

The following account describes the effect of benzimidazole on the differentiation of ectodermal explants prepared from the late gastrulae of *Xenopus laevis*. These effects are compared with the action of benzimidazole on explants not capable of differentiation, prepared from early gastrulae.

EXPERIMENTAL

Fertilized eggs were obtained from the toads by injection of chorionic gonadotropin. Capsules and vitelline membranes were removed, and the embryos transferred to full-strength Holtfreter's solution, where the explants were made. Sterile conditions were maintained during the operations and for the subsequent culture of the explants.

The explants from early gastrulae (stage 10–10½ of Nieuwkoop & Faber, 1956) consisted of the ectoderm covering the blastocoel cavity and excluded the dorsal lip and any invaginated material. The explants from late gastrulae (stage 11½–12 of Nieuwkoop & Faber) were prepared similarly and consisted of the ectoderm covering the remains of the blastocoel cavity and that in contact with the archenteron roof. Care was taken to remove adhering cells of the underlying tissue.

As soon as the explants were made they were placed either in full-strength Holtfreter's solution or in Holtfreter's solution containing 100 μg./ml. of benzimidazole. About half the treated explants were placed in full-strength Holtfreter's solution before transfer to the solution containing the benzimidazole (delayed treatment). This was to allow the formation of a closed external surface. The solutions were buffered either with phosphate or with bicarbonate to a pH of 6·8–7·2. The explants were cultured on a layer of 2 per cent. agar. Under these conditions the concentration of benzimidazole in the test solutions was found to fall, owing to its absorption by the agar. The solutions were renewed

about every 24 hours, giving an effective concentration of benzimidazole between 50 and 100 μg./ml.

The explants were removed and fixed in Bouin's mixture after approximately 48, 60, and 72 hours. Serial sections of 5–7 μ were cut. The sections were stained either with pyronine and methyl green or with haematoxylin.

RESULTS

The number of explants made, their types, the number which survived, and the extent of their differentiation are given in Table 1.

TABLE 1

Differentiation of ectodermal explants from gastrulae of Xenopus laevis

Type	*Duration of culture (hours)*	*No. made*	*No. survived*	*Differentiation*			
				Ectoderm only	*Neuroid*	*Neural*	*Mesenchyme*
Early gastrulae	68	Cont. 26	6	6	0	0	0
		Test 30	7	6	0	0	0
Mid- to late gastrulae	48	Cont. 30	19	9	5	4	0
		Test 32	24	9	5	10	0
		Test (D) 30	25	13	8	4	0
Mid- to late gastrulae	60	Cont. 31	24	1	11	12	5
		Test 33	20	3	10	8	2
Mid- to late gastrulae	72	Cont. 22	22	8	1	12	8
		Test 24	23	7	4	11	9
		Test (D) 15	13	4	1	8	4

Cont. = Holtfreter's solution.

Test = Holtfreter's solution + benzimidazole 50–100 μg./ml.; explants placed in solution immediately.

Test (D) = Holtfreter's solution + benzimidazole 50–100 μg./ml.; explants placed in solution 3 hours after preparation, to allow healing.

Explants were considered to have undergone neuroid differentiation if they possessed an internal mass of cells which was distinct from the surrounding ectoderm (Plate, fig. A). This cell mass was characterized by the following features. It possessed a vague structure, the cells being roughly orientated about the centre of the mass. The cells of the mass were smaller and more heavily stained than those of the epidermis. Mitoses were observed frequently in the neuroid mass; in the epidermis mitoses were rare. Neural differentiation, which is invariably of the archencephalic type in these explants, was considered to be present when distinct structures composed of neuroid cells were seen (Plate, fig. E).

The explants made from the early gastrulae did not undergo differentiation. In these explants the survival was poor, both in the tests and in the controls. After 68 hours' culture no differences could be detected between the treated and untreated explants, either in terms of survival or in histological appearance.

In addition to the explants from the early gastrulae, about a third of the explants from the middle to late gastrulae failed to differentiate. In these cases, too, no difference could be detected between the test and control explants.

Benzimidazole neither prevented neural differentiation nor did it noticeably affect the survival of explants prepared from middle to late gastrulae. This is clearly shown by the facts which are summarized in Table 2. Although benzimidazole did not prevent differentiation, the neural tissue which was formed

TABLE 2

Effect of benzimidazole on the survival and differentiation of Xenopus *ectodermal explants from mid to late gastrulae*

(Cultured 48–72 hours; figures are percentages)

	Benzimidazole 50–100 *μg./ml.*	*Controls*
Survived	78	79
Neuroid differentiation	28	26
Neural differentiation	39	43

TABLE 3

Location of damaged cells

Xenopus ectodermal explants mid- to late gastrulae. Benzimidazole 50–100 μg./ml.

	48		60		72	
Duration of culture (hours):	*Cont.*	*Test*	*Cont.*	*Test*	*Cont.*	*Test*
No. of explants showing neuroid–neural differentiation:	9	27	23	18	13	24
Cell damage: Between neuroid mass and ectoderm	0	5	13	14	0	10
Cell damage: Closely investing neuroid mass	0	1	0	7	0	6
Cell damage: Scattered in neuroid mass	0	6	1	15	0	21

was clearly affected by the treatment. The location of the damaged cells in the explants which had undergone neuroid or neural differentiation is given in Table 3. Cells which were presumably dead or dying were revealed by abnormally intense staining. Such cells were localized mainly in three places. They were seen between the differentiating neural mass and the ectoderm, closely investing the neuroid mass, and scattered throughout the neuroid or neural structure itself. Abnormally stained cells were also seen in the control explants, but they were not very numerous and tended to be scattered throughout the sections. After about 48 hours' culture about a third of the treated explants contained damaged cells. At 60 hours nearly all the treated explants were

affected. At 72 hours, in addition to the neural tissue, the ectomesenchyme was affected.

The most striking pattern of cell damage seen in some of the treated explants was a ring of material heavily stained with pyronine and closely investing the differentiating neural mass. This material appeared to consist of two components. One was a layer of shrunken cells between the neural structure and the ectoderm. In the living material this component was probably a layer of dead or dying cells surrounding the spherical mass of the differentiating neural cells, lying beneath the adhesive gland. The second component consisted of the distal portion of the cytoplasm of the cells on the outside of the neural structure.

In corresponding areas of control sections the border between the ectoderm and the differentiating neural mass was sometimes seen to be demarcated by a thin line of pyronophilic material, consisting of the borders of the cells on the periphery of the neural mass. Compared with the treated sections, however, this area was much less intensely stained. The contrast in the appearance of these areas in test and control sections is seen in figs. A, B, C, D of the Plate.

After 48 and 60 hours' culture, sections of many of the treated explants revealed pycnotic and heavily stained cells in the developing neural tissue. After 72 hours the neural tissue of the treated explant showed extensive damage (Plate, figs. E, F). In addition to cells containing pycnotic nuclei, these contained many cells in which the nuclei were abnormally large and lightly stained. These large nuclei are shown in fig. G of the plate, where they may be compared with nuclei in a corresponding area of a control explant (Plate, fig. H).

The cytological appearance of the epidermis appeared to be identical in the treated and untreated explants. After 60–70 hours the ectoderm taken from middle to late gastrulae became ciliated. At 72 hours the ciliated epidermis was sufficiently developed to propel some of the explants over the agar base in the culture dishes. Movement of this kind was shown by both the treated and untreated explants.

DISCUSSION

The results indicate that the neural tissue and the ectomesenchyme which form in the explants are selectively damaged by benzimidazole, the effect being very similar to that seen in whole embryos. The epidermis does not appear to be affected by the treatment. Explants which have been in contact with benzimidazole for as long as 3 days possess epidermis which cannot be distinguished either histologically or functionally from that of the controls.

It is especially interesting to note that the effect of benzimidazole is only revealed as cell damage after about 2 days, when about half the explants prepared from middle to late gastrulae have begun to differentiate. Only about a quarter of these explants show signs of cell damage. This delayed effect may be due either to a slow build-up of a toxic concentration of benzimidazole inside the explant, or to the absence of susceptible cells when the explants were made.

A delayed effect seems to be ruled out from observations on intact embryos, where toxic effects are observed after about 24 hours. Bearing in mind that explants are very much smaller than entire embryos, it seems reasonable to assume that the cells of the neuroid mass in the explants will develop in a concentration of benzimidazole which would have proved lethal to embryos.

When differentiation starts in the explants neither the formation of the neuroid mass nor its further development into a neural structure of some kind is prevented by benzimidazole. However, cytological damage is observed soon after differentiation has begun and becomes widespread as differentiation proceeds. A marked feature of the differentiation areas is the presence of numerous mitoses. Cell division proceeds in the presence of benzimidazole. A count of the number of mitoses in the test and control explants at 60 hours indicated that, at this stage, mitosis was unimpaired in the treated explants. At 72 hours, on the other hand, there were fewer mitoses in the treated explants than there were in the controls. This is almost certainly a secondary effect.

In some of the treated explants abnormally high concentrations of pyronophilic material were seen in the cytoplasm of the neuroid cells on the periphery of the neuroid mass. This type of cytoplasmic staining does not appear to be related specifically to the action of benzimidazole. Waddington (1958) has described the formation of strongly pyronophilic lumps of material in the cytoplasm of the neural cells of amphibian embryos treated with tri-ethyl melamine and myleran, and he suggests that such globules may indicate an overproduction of ribonucleic acid.

At 60 hours the most characteristic abnormality seen in the treated explants was simply shrunken, pyronophilic, cells. At 72 hours, however, in addition to pycnotic cells, many of the cells of the neural tissue appeared enlarged and contained large nuclei which were poorly stained. It must be emphasized that this latter cytological appearance occurs after the pycnotic abnormalities have become well established. Thus any suppression of cell division and consequent enlargement of interphase nuclei must be regarded, in this case at least, as secondary and possibly the result of toxic effusions from neighbouring moribund cells. These cytological appearances reveal only that some of the cells were killed, either directly or indirectly, by benzimidazole. We learn nothing about the original biochemical lesion.

More significant than the pathological appearance of individual cells is the overall pattern of cell damage. In the treated explants the highest concentration of damaged cells undoubtedly surrounds the neural mass. This is indicated in the earlier stages by the appearance of a pyronophilic ring surrounding the neural mass and later by a mass of degenerating cells lying between the epidermis and a poorly organized neural structure. A pattern of cell damage of this kind is consistent with the idea that cells in the process of differentiation, following an initial phase of mitoses, are especially sensitive to the action of benzimidazole.

The biological effects of benzimidazole and its derivatives need to be

interpreted with extreme caution. The compounds have diverse effects on a number of apparently very different systems. Some derivatives produce general toxic effects. For instance, the dichloro-ribazole derivative reduces oxygen uptake and causes cell damage in the chorio-allantoic membrane of the chick (Tamm, 1956). Administered to mammals, benzimidazole acts as a muscle depressant (Goodman, Gillman, & Hart, 1943; Goodman & Hart, 1944). The swimming activity of amphibian larvae is quickly inhibited by benzimidazole and its alkyl derivatives (Billett, 1958). Further, there is some evidence to suggest that benzimidazoles are metabolic antagonists of vitamin B_{12} (Arscott, Shorb, & Boggs, 1955).

However, the concentrations of benzimidazole and its derivatives required to produce these general toxic and pharmacological effects are relatively high, and there is good reason to believe that the benzimidazoles can act selectively if used at sufficiently low concentrations. Tamm and his colleagues have shown convincingly that benzimidazole can reduce the yield of influenza virus grown on chick allantois without noticeable effects on the uninfected host cells. In the case of *Xenopus* explants, culture in benzimidazole results in gross cytological damage in the central differentiating mass. A high concentration and rapid turnover of ribonucleic acid appears to be a feature of differentiating cells, and very similar conditions probably exist in cells supporting the growth of RNA-containing viruses. In both cases the deleterious action of benzimidazole and its derivatives could be due to one and the same cause, a selective disruption of RNA metabolism.

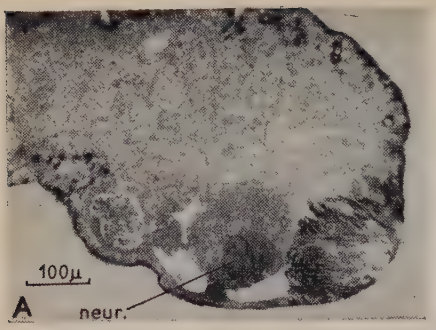
SUMMARY

The survival of ectodermal explants taken from early to late gastrulae of *Xenopus laevis* does not appear to be affected by culturing them, for up to 3 days, in solutions containing 50–100 μg./ml. of benzimidazole. This compound does not prevent neural differentiation. Cell damage only becomes apparent after about 48 hours when it is confined to the differentiating neural tissues and the surrounding cells. The histological appearance of the ectoderm cells and the fact that they develop functional cilia suggest that they are not affected by the treatment. The results suggest that tissue in the process of differentiation is highly susceptible to benzimidazole. This susceptibility may be a consequence of the high rate of turnover of RNA in differentiating tissue.

RÉSUMÉ

Effet du benzimidazole sur la différenciation de fragments d'ectoderme prélevés sur des gastrulas de Xenopus laevis

La survie des fragments d'ectoderme prélevés chez *Xenopus laevis* depuis le début jusqu'à la fin de la gastrulation ne paraît pas affectée par leur culture, dans les limites de trois jours, dans des solutions contenant 50 à 100 μgr. de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neur.
A


bas. r.
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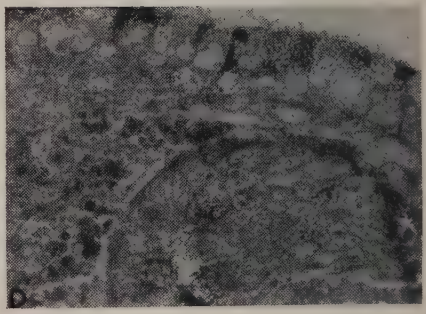
C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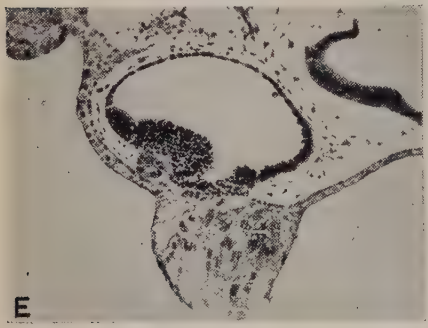
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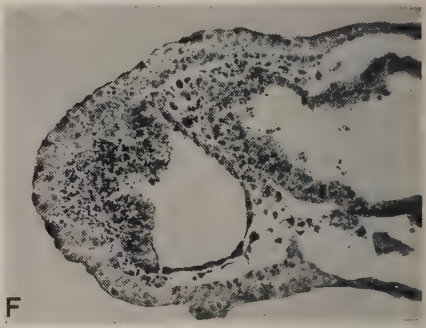
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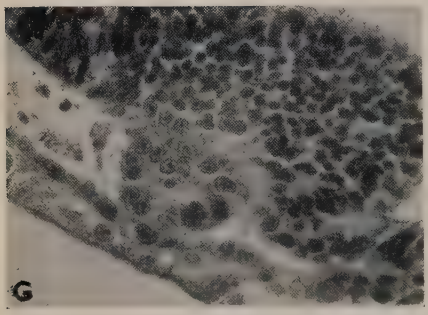
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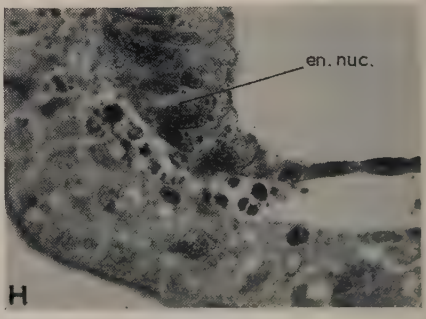
en. nuc.
H

benzimidazole par ml. Ce composé n'empêche pas la différenciation neurale. Des lésions cellulaires ne deviennent apparentes qu'après environ 48 heures et sont alors limitées aux tissus neuraux en différenciation et aux cellules voisines de ceux-ci. L'aspect histologique des cellules de l'ectoderme, et le fait qu'elles se garnissent de cils fonctionnels suggèrent qu'elles ne sont pas affectées par le traitement. Les résultats suggèrent qu'un tissu en voie de différenciation est très sensible au benzimidazole. Cette sensibilité peut être une conséquence du métabolisme intense de l'ARN dans le tissu en différenciation.

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We are deeply indebted to Professor C. H. Waddington, F.R.S., for his interest in our work and for his help during the preparation of the manuscript. We are most grateful to Mr. C. Atherton for taking the photomicrographs.

REFERENCES

ALLFREY, V. G., MIRSKY, A. E., & OSAWA, S. (1957). Protein synthesis in isolated cell nuclei. *J. gen. Physiol.* **40**, 451–90.

ARSCOTT, G. H., SHORB, M. S., & BOGGS, U. (1955). Effect of vitamin B_{12} and antimetabolites of vitamin B_{12} on chick hatchability. *Poult. Sci.* **34**, 986–8.

BILLETT, F. (1958). The temporary inactivation of newt larvae by benzimidazole and its alkyl derivatives. *Experientia*, **14**, 99–100.

—— & PERRY, M. M. (1957*a*). The effect of certain alkyl derivatives of benzimidazole on the embryos of *Xenopus laevis*. *Proc. roy. Phys. Soc. Edinb.* **25**, 59–62.

—— —— (1957*b*). The effect of certain alkyl derivatives of benzimidazole on chick embryos grown *in vitro*. *Proc. roy. Phys. Soc. Edinb.* **26**, 15–18.

GOODMAN, L., GILLMAN, A., & HART, N. (1943). Preliminary investigations on the pharmacology of benzimidazole. *Fed. Proc.* **2**, 80.

—— & HART, N. (1944). Further studies on the central nervous system action of benzimidazole. *Fed. Proc.* **3**, 73.

LIEDKE, K. B., ENGLEMAN, M., & GRAFF, S. (1954). The selective response of amphibian embryos to benzimidazole and benzotriazole derivatives. *J. exp. Zool.* **127**, 201–19.

NIUEWKOOP, P. D., & FABER, J. (1956). *Normal Table of* Xenopus laevis (Daudin). Amsterdam: North Holland Publishing Co.

SLONIMSKI, P. P. (1954). Sur l'inhibition de la croissance de la levure par le benzimidazole: mécanisme de la réversion de l'inhibition. *Ann. Inst. Pasteur*, **87**, 404–13.

TAMM, I. (1956). Selective chemical inhibition of influenza virus multiplication. *J. Bact.* **72**, 42–53.

—— (1957). Ribonucleic acid synthesis and influenza virus multiplication. *Science*, **127**, 1235.

—— (1958). Selective inhibition of virus multiplication. In *The Strategy of Chemotherapy*, ed. Cowan, S. T. & Rowatt, E. Camb. Univ. Press.

—— FOLKERS, K., SHUNK, C. H., & HORSFALL, F. L. (1953). Inhibition of influenza virus multiplication by alkyl derivatives of benzimidazole. *J. exp. Med.* **98**, 245–59.

WADDINGTON, C. H. (1958). A note on the effect of some cytotoxic substances on amphibian embryos. *J. Embryol. exp. Morph.* **6**, 363–4.

—— FELDMAN, M., & PERRY, M. M. (1955*a*). Some specific developmental effects of purine antagonists. *Exp. Cell Res.*, Suppl. 3, 366–80.

—— & MANCUSO, V. (1955*b*). The effects of some purines and purine analogues on ascidian embryos. *Exp. Cell Res.* **9**, 369–71.

EXPLANATION OF PLATE

FIG. A. Section of explant cultured for 60 hours in Holtfreter's solution. Note neuroid differentiation (*neur.*).

FIG. B. Section of explant cultured for 60 hours in Holtfreter's solution containing 50–100 μg./ml. benzimidazole. Note the basophilic ring (*b.r.*) surrounding the differentiating cell mass.

FIG. C. The section shown in fig. A at a higher magnification, showing the neuroid mass of cells.

FIG. D. The section shown in fig. B at a higher magnification, showing part of the basophilic ring.

FIG. E. Section of explant cultured for 72 hours in Holtfreter's solution, showing neural differentiation.

FIG. F. Section of explant cultured for 72 hours in Holtfreter's solution containing 50–100 μg./ml. benzimidazole, showing poor development of neural structure.

FIG. G. Section shown in fig. E at a higher magnification.

FIG. H. Section shown in fig. F at a higher magnification. Note enlarged nuclei in neural tissue (*en. nuc.*).

All sections were cut at 5–7 μ. Specimens A, B, C, and D stained with methyl green and pyronine; specimens E, F, G, and H stained with haematoxylin.

(*Manuscript received 6 : iv : 60*)

The Development of Anucleolate Embryos of *Xenopus laevis*

by H. WALLACE[1]

From the Department of Zoology and Comparative Anatomy, University Museum, Oxford

WITH PLATE

A MUTATION of *Xenopus* has been found which reduces the maximum number of nucleoli per diploid nucleus from 2 in the wild-type to 1 in heterozygotes. Homozygous mutants possess no true nucleoli, hence being termed *anucleolate*. They do, however, possess pyronine-staining intranuclear organelles that are smaller and more numerous than typical nucleoli. The mutation can be considered as a recessive larval lethal, unlinked to sex: heterozygotes of both sexes are fully viable, but anucleolate larvae die at about the time their sibs begin to feed. These points have been recorded in two preliminary reports (Elsdale, Fischberg & Smith, 1958; Fischberg & Wallace, 1960). The development of anucleolate embryos is described here as a basis for the design of an experimental analysis of the ways in which the mutation acts. The description rests mainly on the progeny of a single mating of heterozygous toads, conforming with notes made on other such matings, from which additional data are drawn when required. As no developmental differences have been detected between the wild-type and heterozygotes, they are treated together as nucleolate ($+n$) controls, to which the anucleolate (*On*) embryos are compared. Nieuwkoop & Faber's (1956) stages of normal development are used here to indicate age, made independent of temperature. After stage 40, the retarded *On* larvae are assigned to the stage of their controls.

EXTERNAL DESCRIPTION

Camera lucida drawings and records of some physiological and morphological characters have been made on a random sample of embryos from stage 28 onwards. The characters studied are summarized in Table 1. Successive drawings of a typical *On* tadpole and a $+n$ control are shown in Text-fig. 1.

Anucleolate embryos hatch during the same period as controls (stage 35/36). No differential mortality occurs before this. Soon after hatching, control larvae swim upwards and suspend themselves, by means of the cement gland secretion, from the water surface or the sides of the container. *On* larvae are relatively

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TABLE 1

Comparison of characters of On *and control larvae, from observations at 12-hour intervals*

(o = present in *On*; + = present in +*n* larvae)

Days at 18–19° C.:	3	4	5	6	7	8	9	10	11	12	13
Nieuwkoop stage:	32 33	35 37	40 41	42 43	44 45	46 46	46 46	47 47	47 47	47 47	47 47
Ciliary motion	o+ o+	o+ o+	..	..	..	..	..	..	..	..	..
Muscular twitch	o+ o+	o+ o+	o+ o+	o+ o+	o+ o	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ +
Regular swimming	..	..	..	..	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Gulping	..	..	..	..	..	+ +	+ +	+ +	+ +	+ +	+ +
Eye-twitch	..	..	..	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Heart-beat	..	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ +
Blood circulation	..	.. o+	o+ o+	o+ o+	o+ +	+ +	+ +	+ +	+ +	+ +	+ +
Sucker secretion	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o o	o o	o o	o o	o o	o ..
Melanophores	..	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ +
Erythrocytes	..	..	..	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ +
Otolith	..	..	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ o+	o+ +
Head oedema	..	..	..	..	..	.. o	o o	o o	o o	o o	o ..
Heart oedema	..	..	..	..	o o	o o	o o	o o	o o	o o	o ..
Anal oedema	..	..	..	o o	o o	o o	o o	o o	o o	o o	o ..
On survival as a percentage of 25 larvae	100	..	..	..	..	..	100	96 96	84 56	20 12	4 0
+*n* survival as a percentage of 25 larvae	100	..	..	..	..	..	100	..	..	..	100

inactive in this respect (Table 2). This difference plays an important part in the design of suitable culture conditions: *On* larvae must be isolated after hatching. Otherwise they are rapidly infected by the decaying eggs found at the bottom of most mass cultures.

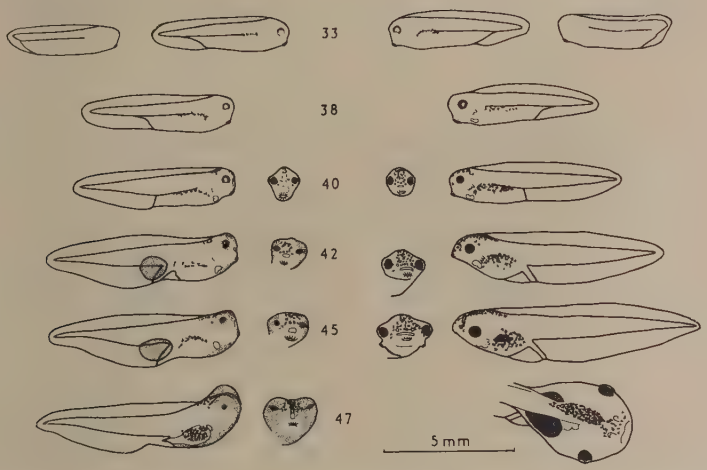

FIG. 1. Successive drawings of a typical *On* larva (on left) and a control larva (on right). The numbers refer to stages of normal development. Cranial and trunk melanophores and the retinal pigment are shown in black; other organs in outline only. Oedemata are stippled, and they are encircled where protruding from the body contour.

TABLE 2

Suspension of newly hatched larvae

	Controls	*Anucleolate*
Became suspended in 3½ hours . . .	579	38
Remained at bottom of tank for 3½ hours .	91	173
TOTAL	670	211

A sample of newly hatched larvae were shaken to the bottom of a tank containing 2 inches of water. Those that rose and became suspended were collected periodically, and diagnosed at stage 40. The heterogeneity ($\chi^2_{(1)} = 354{\cdot}6$; $P < 0{\cdot}001$) shows that anucleolate larvae are relatively inactive. The proportion of anucleolate larvae in the sample agrees with a 3:1 ratio ($\chi^2_{(1)} = 0{\cdot}412$; $P > 0{\cdot}5$); no differential mortality has occurred.

Anucleolate larvae are externally indistiguishable from their sibs up to stage 39. After this, the jaw-growth of control larvae pushes the cement gland to a high anterior position (Text-fig. 1). The cement gland of *On* larvae remains in a ventral position, very slightly in front of the eyes. This is the earliest distinguishing morphological character found. Assuming good culture conditions and the absence of microcephalic controls, it is quite reliable by stage 40. After stage 40, *On* larvae become noticeably retarded in such aspects of growth as

tail-expansion, head-enlargement, gut-coiling, heart-growth, and the spreading of melanophores over the head and flank. As *On* larvae show few signs of life (the heart-beat may escape detection), they were considered dead only when they had decayed so far that the oedemata had collapsed and their eyes had swollen (Table 1; relative survival). Some might have survived longer in more sterile culture but they were obviously incapable of feeding. The survival of all the more active unfed sibs shows that starvation was no cause of death.

Two observations in Table 1 require comment. The blood circulation of *On* larvae at first seems completely normal and can even be traced through the rudimentary external gills. Later, the blood corpuscles settle in such areas as the pronephric sinus and subcaudal vein. Corpuscular movement was very rarely observed after stage 44, although the heart continues to beat. This may probably be attributed to the oedematous condition of the *On* larvae and to an inferior pumping action of the heart. When motionless stage 40 tadpoles were compared, *On* larvae showed a subnormal rate of heart-beat. The mean time of 100 beats recorded at 23–24° C. was $55{\cdot}2 \pm 0{\cdot}639$ seconds for 20 control larvae, and $71{\cdot}0 \pm 1{\cdot}395$ seconds for 20 *On* larvae. A *t*-test shows this difference to be significant ($t = 11{\cdot}356$ for 38 degrees of freedom, $P < 0{\cdot}001$). The cement gland normally ceases to secrete at about stage 46 and is degenerating by stage 47. The expected degeneration of the cement gland of *On* larvae is delayed; they can be attached to the water surface until shortly before death.

Elsdale *et al.* (1958) mention, as characteristic of *On* larvae, that the tail-tip is crumpled and bent ventrally. I suspect this to be an effect of suboptimal culture conditions, but there is a tendency for *On* larvae to show this character. From three samples of stage 43 *On* larvae (from successive matings) 7 per cent., 20 per cent., and 48 per cent. showed a ventral flexure of the tail-tip.

MICROSCOPIC EXAMINATION

Groups of embryos were fixed in Zenker at various times corresponding to normal stages of development of the controls (with *On* embryos of the same age). They were cut in 8μ transverse sections and stained with Jordan & Baker's (1955) mixture of pyronine and methyl green. The description below is based upon the following numbers of *On* embryos: 1 at stage 28, 2 at stage 29/30, 4 at each of stages 31, 35/36, 39, 40, 42, 45, 47; and an equal number of controls at each stage.

Central nervous system

The brain of *On* embryos is normal in shape and size up to stage 31, but already contains an excessive number of cells with pycnotic nuclei (Text-fig. 2). These fall into the lumen of the brain, where cell debris is found between stages 35/36 and 42. By stage 42 the brain has partially collapsed and the organization of nuclei and axons is irregular. Most of the pycnotic cells have been lost by stage 45. No further significant pycnosis occurs until death. Mitosis occurs at all

stages in the cells lining the lumen of the brain, but is less common in the later stages of *On* larvae (Text-fig. 2). This is partly due to the fact that a proliferation of the mid-brain floor occurs at stages 40, 42, and 45 only in controls. The spinal cord of *On* larvae shows the same syndrome as the brain, although delayed in its caudal region. Pycnotic cells appear and are discharged into the lumen of the cord. The formation of both dorsal and ventral nerve roots is delayed. The

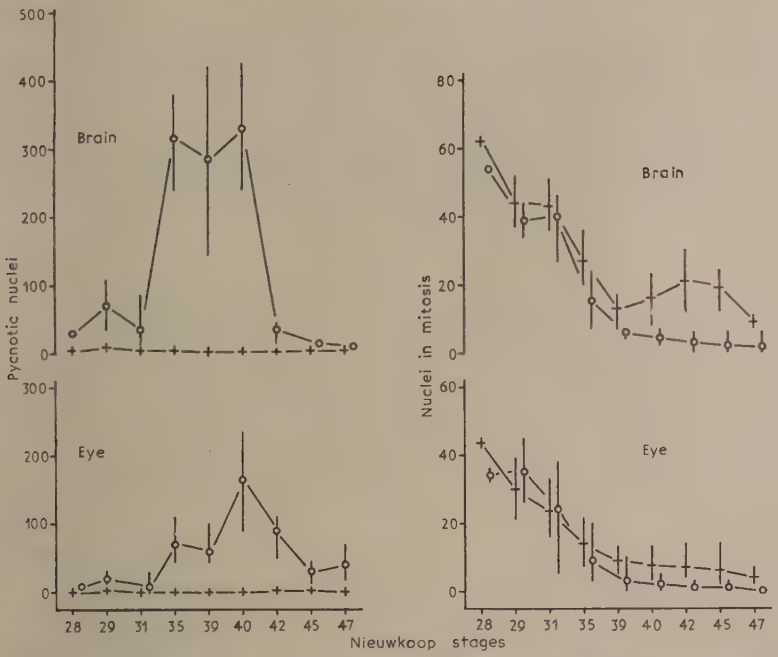

FIG. 2. Counts of pycnotic nuclei and mitotic figures, made on 3 adjacent sections of the midbrains and eye-cups. The mean values and ranges of the counts are shown against the developmental stage. *o* — *o*, anucleolate larvae; + — +, control larvae.

auditory ganglion possesses some pycnotic cells in its early stages. The primary optic vesicle is of normal shape and size, and invaginates normally (stages 31–35/36). It is also subject to pycnosis, cell debris being discharged into the cavity between the eye-cup and the lens. The retina collapses on to the lens at about stage 39. Differentiation of the retinal layers proceeds slowly and is disturbed by patches of pycnotic cells. Counts of mitotic figures and of pycnotic nuclei in the eye-cup are given in Text-fig. 2.

Other ectodermal derivatives

The ectoderm is competent to form nasal and auditory placodes and the lens. The epidermis contains the normal large unicellular glands after stage 40: apart from the wrinkles caused by subsided oedemata, it seems to be normal. The

cement gland has a perfectly normal structure and is retained throughout life. The lens is initially normal, forming a mass of primary fibres (stage 35/36). There is little or no secondary growth of the lens by addition of fibres from the retinal side of the lens. Also, the lens is permanently attached to the overlying epidermis—which is only a transient phase of normal lens development. Pycnotic nuclei have been observed only occasionally in this tissue. The auditory vesicle and nasal placode both contain pycnotic cells at stages 28–42. The development of both is retarded. The visceral arch ectomesenchyme appears as a rather looser tissue than in controls. Both the jaw cartilage and transverse mandibular muscle anlagen contain pycnotic cells (stages 35/36–42). After stage 39 the condensation of these anlagen is distinctly retarded; some differentiation into muscle fibres and cartilage is followed ultimately by the formation of some cartilage in the gill-arches. These ectomesenchymal structures are inferior to those developed by controls at stage 42.

Mesoderm

The development of the trunk notochord and somites is identical to that of controls. The heart has a normal appearance until stage 40 but remains a simple twisted tube, while the heart of $+n$ larvae develops a thicker myocardium, ventricular trabeculae (stage 42), and two auricles by stage 47. The *On* heart contains no pycnotic tissue. The pronephros develops normally up to stage 39, when it has three ciliated funnels, a slightly coiled collecting tube, and a duct opening to the cloaca. Little further coiling of the collecting tube occurs, and it is often swollen into a series of bladders. By stage 40 the pronephric sinus is gorged with erythrocytes and cell debris. The latter is found within the lumen of the pronephros and its duct. Presumably the cell debris comes from other tissues and is being excreted: the pronephric tissue itself appears to be perfectly healthy. No divergence from normality has been found in the lateral plate mesoderm.

Endoderm

The resorbtion of the postanal gut is not markedly delayed, but intestinal coiling is retarded; only a single gut loop is achieved, corresponding to that of stage 42–43 controls. The mouth opens at the usual time (stage 40). The cells of the pharyngeal floor of control larvae proliferate (stages 35/36–40) to form a medial area of subepithelial columnar cells, which constitute the bulk of the 'primitive tongue', and three gill-strands on either side. A similar proliferation is found at the same time in *On* larvae but is accompanied by pycnosis, beginning at stage 35/36 and judged to be most intense at stage 42. No columnar cells are formed, and the break-through of the gills is delayed. The larynx and lung diverticula appear late and grow slowly. The liver eventually develops lacunae containing erythrocytes and some pycnotic cells, which may be found in all parts of the blood-stream and so are not particularly associated with the liver.

The various parts of the gut can be recognized by their position, with the exception of the pancreas, which has not been identified. The stomach, duodenum, and intestine show little differentiation, and a moderate amount of pycnosis that is not strikingly greater than may be encountered in control tadpoles.

DISCUSSION

These observations lead to the conclusion that *On* embryos develop at a normal rate until about the time of hatching, after which they become increasingly retarded and abnormal. Their development is not completely arrested, at least until they correspond in appearance to stage 42 controls.

The incidence of pycnosis suggests that it is related to cellular differentiation. In the midbrain and eye-cup, pycnosis reaches its maximum intensity between stages 35/36 and 40 (Text-fig. 2). The majority of cells survive this period and are able to differentiate. Thus the pycnosis is quite distinct from that which affects moribund tadpoles. There are two observations which support the contention that this neural pycnosis is not correlated with abnormal cell-division. Firstly, all phases of mitosis are seen and appear completely normal. Secondly, at the stages considered, cell-division is virtually restricted to cells lining the lumen of the brain and spinal cord (or its vestige in the eye-cup), yet pycnosis is found in all parts of the neural tissues. The rather low mitotic counts of *On* brain and eye-cup (Text-fig. 2) may result from the reduced number of cells, or from a lower rate of mitosis that might be expected from the generally retarded development.

Although it is difficult to distinguish between primary and secondary abnormalities on the basis of a purely descriptive study, it seems clear that the abnormality of the central nervous system is caused neither by defective induction nor by a deficient circulation. The central nervous system of tail-bud stages has a normal conformation (unlike that of typical microcephalic larvae), while neural pycnosis is common before stage 33/34 when the heart normally begins to beat. The defective lens development may be dependent on the abnormal eye-cup, as has been demonstrated to be the case in several amphibians (Balinsky, 1957). The pharyngeal pycnosis and atypical differentiation could also be a secondary abnormality; Okada (1957) has shown that the pharyngeal differentiation of urodeles is induced by ectomesenchyme. The retarded development and growth of other organ systems may result from the inferior blood circulation.

SUMMARY

1. Anucleolate *Xenopus* embryos develop into normal neurulae and tail-bud stages. After hatching, the development of all their organs is retarded. Only then can anucleolate larvae be recognized by such external characters as relatively small brains, eyes and jaws, and anal (followed by other) oedemata.

2. An abnormally large number of pycnotic nuclei occur in the central

nervous system, eye-cup, auditory vesicle, nasal placode, and visceral arch ectomesenchyme. The majority of cells of these tissues are not affected by pycnosis, and are able to differentiate.

3. The mesodermal organs are not affected by pycnosis. Their development is retarded after hatching.

4. Of the endodermal organs, severe pycnosis is encountered only in the pharyngeal floor, which differentiates atypically.

5. It is suggested that the primary abnormality of these mutants concerns the central nervous system, neural crest, and sensory ectodermal derivatives. The abnormal development of the lens and pharynx may be caused by abnormal secondary inductions.

RÉSUMÉ

Le développement des embryons anucléolés de Xenopus laevis

1. Les embryons anucléolés de *Xenopus* se développent normalement jusqu'au stade de la neurula et du bourgeon caudal. Après l'éclosion, le développement de tous les organes est retardé. C'est alors seulement qu'il est possible de reconnaître les larves anucléolées à la taille relativement petite de leur cerveau, de leurs yeux, et de leurs mâchoires, et à la présence d'oedèmes anaux (bientôt accompagnés d'autres oedèmes).

2. On trouve un nombre anormalement grand de noyaux pycnotiques dans le système nerveux central, la cupule optique, la vésicule auditive, les placodes nasales, et l'ectomésenchyme des arcs viscéraux. La majorité des cellules de ces tissus n'est pas touchée par la pycnose et se différencie normalement.

3. Les organes mésodermiques ne sont pas affectés par la pycnose. Leur développement est retardé après l'éclosion.

4. Seul, parmi les organes endodermiques, le plancher pharyngien est sévèrement atteint par la pycnose. Sa différenciation est atypique.

5. Il semble que les anomalies primaires de ces mutants affectent le système nerveux central, la crête neurale, et les organes des sens d'origine ectodermique. Il est possible que le développement anormal du cristallin et du pharynx soit dû à des inductions secondaires anormales.

ACKNOWLEDGEMENTS

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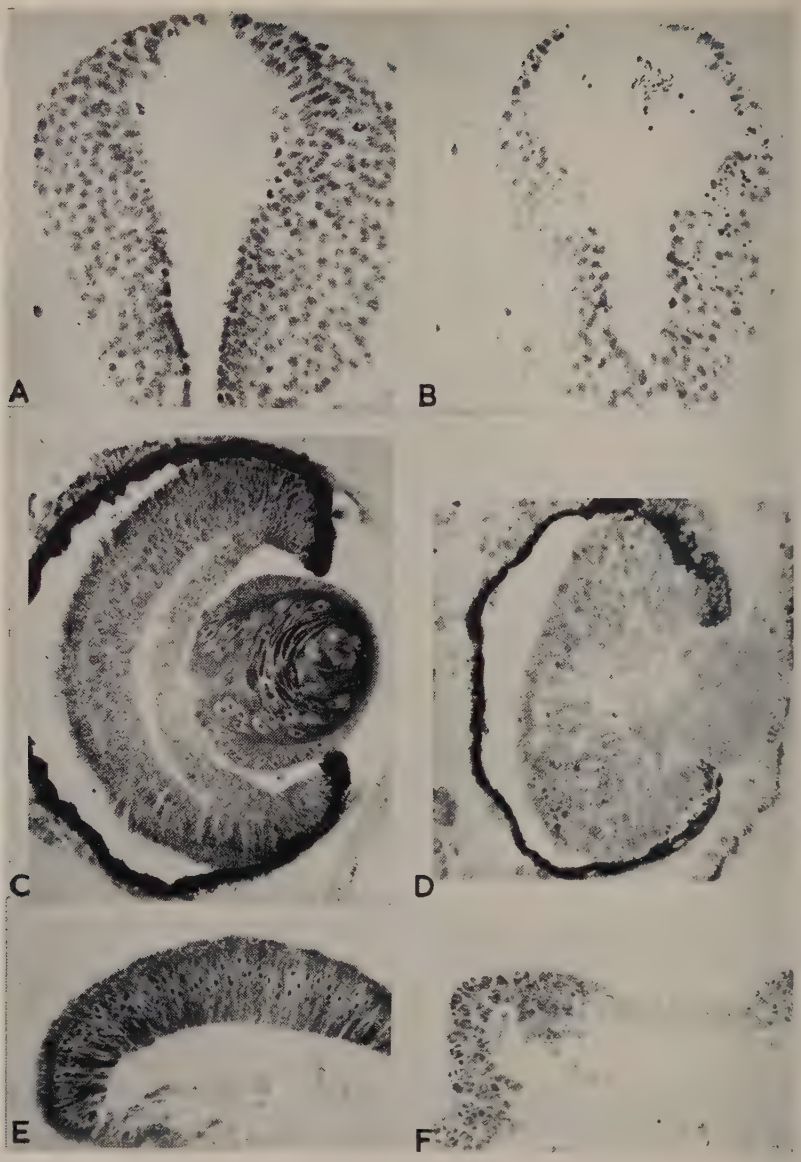
REFERENCES

Balinsky, B. I. (1957). On the factors determining the size of the lens rudiment in amphibian embryos. *J. exp. Zool.* **135**, 255–99.

Elsdale, T. R., Fischberg, M., & Smith, S. (1958). A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp. Cell Res.* **14**, 642–3.

Fischberg, M., & Wallace, H. (1960). A mutation which reduces nucleolar number in *Xenopus laevis*. In *The Cell Nucleus*, ed. B. Holmes. London: Butterworth's Scientific Publications.



H. WALLACE

JORDAN, B. M., & BAKER, J. R. (1955). A simple pyronine/methyl green technique. *Quart. J. micr. Sci.* **96**, 177–9.

NIEUWKOOP, P. D., & FABER, J., ed. (1956). *Normal table of* Xenopus laevis (*Daudin*). Amsterdam: North Holland.

OKADA, T. S. (1957). The pluripotency of the pharyngeal primordium in urodelan neurulae. *J. Embryol. exp. Morph.* **5**, 438–48.

EXPLANATION OF PLATE

FIG. A. Midbrain of stage 40 control larva, showing cell-division but no pycnosis.

FIG. B. Midbrain of stage 40 *On* larva, showing pycnotic nuclei, cell debris, and one metaphase.

FIG. C. Eye and lens of stage 40 control larva, showing one pycnotic retinal cell.

FIG. D. Eye and lens of stage 40 *On* larva, showing retinal pycnosis and the poor development of both tissues.

FIG. E. Pharyngeal floor of stage 47 control larva, showing columnar cells of the 'primitive tongue'.

FIG. F. Pharyngeal floor of stage 47 *On* larva, showing the atypical cellular differentiation.

(FIGS. A, B—feulgen stain, green filter; dark nuclei. Figs. C, D, E, F—pyronine–methyl green stain, green filter; dark nucleoli.)

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La culture *in vitro* de l'épithélium germinatif isolé des gonades mâles et femelles de l'embryon de Canard

II. Influence de la médullaire sur la différenciation de l'épithélium germinatif

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AVEC TROIS PLANCHES

INTRODUCTION

DANS un travail antérieur en collaboration avec Wolff (1959), nous avions étudié le comportement, en culture *in vitro*, de l'épithélium germinatif isolé des gonades embryonnaires de Canard. Nous avions montré que l'épithélium germinatif est orienté vers la différenciation mâle et femelle, au stade où s'ébauche la différenciation sexuelle dans l'embryon. Cultivé à l'état isolé, il donne naissance, chez le mâle, à des cordons médullaires qui évoluent en cordons testiculaires. Chez la femelle, il évolue en un cortex ovarien très épais qui entoure des nids à ovogonies. Par conséquent, à lui seul, l'épithélium germinatif donne des formations analogues à celles que développe une gonade entière pourvue de sa médullaire.

Par contre, si l'épithélium germinatif est prélevé à un stade antérieur à la différenciation sexuelle, il se montre incapable de se différencier, quel que soit son sexe. La médullaire paraît donc nécessaire aux jeunes stades du développement pour assurer la différenciation sexuelle normale.

Ce résultat nous a amené à éprouver l'action de la médullaire sur l'épithélium germinatif et à poser la question suivante: en présence d'une médullaire de sexe opposé, l'épithélium germinatif se différencie-t-il suivant son sexe génétique ou suivant celui de la médullaire?

Nous exposons ici les résultats de deux séries expérimentales: 1) l'association d'épithélium germinatif indifférencié et de médullaire femelle, et 2) l'association d'épithélium germinatif indifférencié et de médullaire mâle.

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MATÉRIEL ET MÉTHODES

L'épithélium germinatif a été prélevé sur les glandes génitales gauches d'embryons de Canard âgés de 5 à 9 jours d'incubation (stades 17 à 21 de Koecke).

Les médullaires ont été prélevées sur les glandes génitales gauches et droites d'embryons de Canard et de Poulet âgés de 9 à 13 jours d'incubation. Nous nous sommes adressés aux races Khaki Campbell et Leghorn 'blanche'.

Culture in vitro *et préparation des associations*

Les associations ont été cultivées *in vitro* pendant 5 à 7 jours selon la méthode de culture en milieu standard que nous avons mise au point avec Wolff en 1952. Le sexe génétique de l'épithélium germinatif, prélevé au stade de l'indifférence sexuelle, est révélé par la différenciation de la gonade droite que l'on cultive séparément.

La séparation du constituant cortical ou médullaire d'une gonade est faite au moyen de la technique à la trypsine diluée, élaborée par Moscona (1952).

Les gonades sont prélevées en même temps que le mésonéphros auquel elles adhèrent. L'ensemble mésonéphros–gonade est immergé dans une solution de trypsine Difco 1:250 à 2 pour cent dans du liquide de Tyrode, pendant 5 à 15 minutes à 38° C. Après lavage au liquide de Tyrode, il est transporté sur un fond de cire noire. Le mésonéphros est maintenu en place au moyen de fines épingles. On détache l'épithélium de la médullaire à l'aide d'un microscalpel ou d'une aiguille de verre. La ténuité des gonades de 5 à 7 jours rend l'isolement de l'épithélium germinatif plus difficile, de sorte que dans la plupart des expériences, l'épithélium explanté est réduit aux $\frac{2}{3}$ ou aux $\frac{3}{4}$ de sa longueur.

L'épithélium germinatif d'une gonade est mis en contact intime avec la médullaire d'une autre gonade, à la surface du milieu de culture. La médullaire est découpée en plusieurs fragments afin d'entourer complètement l'épithélium. Dans les expériences témoins les médullaires sont remplacées par des fragments de mésonéphros.

Technique histologique

Pour l'étude histologique, les explants, inclus à la paraffine, sont coupés à 5 μ et colorés à l'hématoxyline de Groat.

ASSOCIATIONS D'ÉPITHÉLIUM GERMINATIF INDIFFÉRENCIÉ ET DE MÉDULLAIRE FEMELLE

Les 62 expériences réalisées dans cette série ont donné les combinaisons suivantes: 35 associations d'épithélium germinatif mâle et de médullaire femelle et 27 associations d'épithélium germinatif femelle et de médullaire femelle.

Évolution morphologique des associations

Dans toutes les associations on peut suivre l'évolution morphologique caractéristique des deux constituants en présence. L'épithélium germinatif, au centre, s'élargit et devient translucide; les fragments de médullaire ovarienne qui l'entourent se soudent intimement à l'épithélium et entre eux; leur aspect est opaque et granuleux (Planche 1, fig. 1*a* et *b*). La médullaire de Canard subit une régression importante, identique à celle que l'on observe dans l'ovaire et la gonade droite ♀ de Canard, cultivés *in vitro* (1952*c*). Après 5 jours de culture, il ne reste souvent plus d'elle que de très petits nodules d'aspect granuleux, soudés à l'épithélium germinatif devenu translucide (Planche 1, fig. 1*a*).

Structure histologique des associations

Médullaires femelles

A l'examen des coupes histologiques, on reconnaît aisément les médullaires femelles à leur structure caractéristique, déjà décrites dans des publications antérieures (Wolff & Haffen, 1952 *a*, *b*, *c*, *d*). La médullaire femelle de Canard est constituée d'un réseau conjonctif lâche, contenant des lacunes (Planche 3, fig. 7*a*). La médullaire femelle de Poulet, qui ne subit pas de régression en culture *in vitro*, est formée de cordons plus ou moins testiculaires serrés les uns contre les autres (Planche 2, fig. 6*a*).

Épithélium germinatif

Épithélium germinatif femelle. Les épithéliums génétiquement femelles, isolés de gonades indifférenciées de 5 à 7 jours d'incubation (Planche 1, fig. 2), évoluent au contact de médullaires femelles, en cortex ovariens typiques (Tableau 1*a*). Nous avons déjà décrit cette structure dans un travail antérieur

TABLEAU 1*a*

Associations de médullaire ovarienne de Canard et de Poulet (9 à 13 jours d'incubation) et d'épithélium germinatif femelle de Canard (5 à 7 jours d'incubation)

Âge de l'épithélium germinatif en jours	*Nombre d'explants*	*Différenciation*		*Épithélium disparu ou nécrosé*
		Cortex ovarien	*Indéfinissable*	
$6\frac{1}{2}$ – 7	12	10	1	1
5 – 6	15	12	1	2

(Wolff & Haffen, 1959). Nous la rappelons brièvement. Le cortex est formé d'un assemblage de lames épithéliales repliées sur elles-mêmes qui finissent presque toujours par former des cordons. Chacun de ces cordons ou lames comprend plusieurs assises de hautes cellules étroites et prismatiques à noyaux orientés perpendiculairement à la membrane basale. Les cellules germinales, nombreuses,

sont intercalées entre les cellules épithéliales ou groupées en îlots (Planche 2, figs. 6 *a*, *b* et *c*).

Le fait que, dans les séries témoins, l'épithélium femelle évolue au contact de mésonéphros, de la même manière qu'au contact de médullaire femelle (Tableau 1*b*; Planche 2, fig. 5), nous amène à penser que l'orientation de l'épithélium germinatif vers la différenciation femelle se situe à un stade plus précoce que celui que nous avions déterminé dans un précédent travail (Wolff & Haffen, 1959). L'épithélium germinatif est déjà déterminé à 5 jours d'incubation. Nous confirmerons d'ailleurs ce résultat au chapitre où nous exposerons les résultats d'associations d'épithélium germinatif femelle et de médullaire mâle (pp. 420–1).

TABLEAU 1*b*

Associations d'épithélium germinatif femelle de Canard de 5 à 7 jours d'incubation et de mésonéphros

Âge de l'épithélium germinatif en jours	*Nombre d'explants*	*Différenciation*		*Épithélium disparu ou nécrosé*
		Cortex ovarien	*Indéfinissable*	
$6\frac{1}{2}-7$	3	2	1	—
5 – 6	8	7	—	1

Épithélium germinatif mâle: associations d'épithélium germinatif mâle de 5 à 7 jours avec des médullaires femelles de 9 à 13 jours d'incubation. Parmi les 35 épithéliums mâles, isolés de gonades indifférenciées de 5 à 7 jours et associés à de la médullaire ovarienne, 19 présentent une structure analogue à celle que l'on observe chez les explants femelles (Tableau 2*a*). L'épithélium germinatif

TABLEAU 2*a*

Associations de médullaire ovarienne de Canard et de Poulet (9 à 13 jours d'incubation) et d'épithélium germinatif mâle de Canard (5 à 7 jours d'incubation)

Âge de l'épithélium germinatif en jours	*Nombre d'explants*	*Différenciation*				*Épithélium disparu ou nécrosé*
		Cortex pur	*Cortex + cordons médullaires*	*Cordons médullaires*	*Indéfinissable*	
$6\frac{1}{2}-7$	22	11	4	1	1	5
5 – 6	13	8	—	1	3	1

s'est développé en un véritable cortex dont la structure est identique à celle d'un cortex ovarien (Planche 3, figs. 7 *a*, *b*, *c*). Par conséquent nous pouvons répondre par l'affirmative à la question posée au début du travail, au moins en ce qui concerne l'épithélium mâle: il se différencie suivant le sexe de la médullaire femelle qu'on lui associe. Il convient d'ajouter à ce résultat que les

médullaires femelles de Canard et de Poulet exercent le même effet stimulant malgré leur différence de structure. De plus, aucune différence relative à l'âge ou à la quantité de médullaire femelle en présence n'a pu être mise en évidence; une médullaire de 9 jours est aussi active qu'une médullaire de 13 jours.

Cependant, dans dix cas, l'influence de la médullaire s'est peu ou ne s'est pas manifestée. En effet, 6 épithéliums ne se sont pas différenciés, 4 se sont développés en cortex en même temps qu'ils ont donné naissance à des cordons médullaires mâles.[1] Ces deux derniers résultats rappellent ceux que l'on obtient en associant l'épithélium germinatif à du mésonéphros: explanté au contact d'un tissu neutre, non sexué, l'épithélium mâle évolue soit en un organe constitué d'une médullaire testiculaire, entourée d'un épithélium plus ou moins aplati, soit en un organe de structure uniforme, mais confuse, où aucune différenciation nettement épithéliale ou nettement médullaire ne peut être distinguée (Wolff & Haffen, 1959) (Tableau 2*b*). On remarquera que, dans les

TABLEAU 2*b*

Associations d'épithélium germinatif mâle de Canard de 5 à 7 jours d'incubation et de mésonéphros

Âge de l'épithélium germinatif en jours	*Nombre d'explants*	*Différenciation*		*Épithélium disparu ou nécrosé*
		Cordons médullaires	*Indéfinissable*	
$6\frac{1}{2}-7$	7	3	2	2
5 – 6	9	1	5	3

associations, la différenciation simultanée de cortex et de médullaire s'observe chez les épithéliums âgés de $6\frac{1}{2}$ à 7 jours. Il nous apparaît comme vraisemblable que la médullaire femelle n'a pas, dans ces cas, exercé son effet. Nous pensons que le traitement à la trypsine est à l'origine de l'affaiblissement du pouvoir stimulant de la sécrétion de la médullaire femelle. Mais ces résultats nous amènent aussi à réenvisager la question de la détermination de l'épithélium germinatif mâle. A partir de quel stade l'épithélium germinatif est-il orienté vers la différenciation mâle et jusqu'à quel stade peut-on influencer son évolution normale?

Le fait que l'épithélium germinatif mâle de $6\frac{1}{2}$ jours d'incubation est capable, dans certains cas, de produire des cordons médullaires malgré la présence d'une médullaire femelle qu'on lui associe, semble indiquer que le stade de $6\frac{1}{2}$ jours correspond à celui de son orientation vers la différenciation mâle. Pour répondre à la deuxième partie de la question, les expériences suivantes ont été instituées.

[1] Nous distinguons deux stades dans la différenciation testiculaire: 1) *Cordons médullaires*: cordons en voie d'organisation. Alignement préfigurant l'épithélium sertolien. Les cordons ainsi constitués sont étroits et peu individualisés. 2) *Cordons testiculaires*: épithélium sertolien caractéristique doublé d'une membrane basale très nette, formant des cordons élargis.

Épithélium germinatif mâle: associations d'épithéliums germinatifs mâles de 7½ à 9 jours avec des médullaires femelles de 10 à 11 jours d'incubation. L'épithélium germinatif a été partagé en deux moitiés, dont l'une est associée à de la médullaire femelle, l'autre à du mésonéphros, à titre de témoin (7 cas). Les moitiés d'épithéliums associées à du mésonéphros présentent la structure habituelle: persistance d'une partie de l'épithélium germinatif et différenciation d'une médullaire testiculaire (Planche 3, fig. 8*b*). Les moitiés d'épithéliums associées à de la médullaire femelle présentent une structure analogue. La prolifération de cordons testiculaires n'est pas inhibée, mais la zone épithéliale est plus importante et présente les caractéristiques d'un cortex (Planche 3, fig. 8*a*). Elle est d'autant plus importante que l'épithélium germinatif est plus jeune. On peut penser qu'au moment où l'épithélium germinatif est orienté vers la différenciation mâle, il est difficile d'enrayer la prolifération de cordons testiculaires. On peut encore agir partiellement sur lui en stimulant les formations corticales et ceci au moins jusqu'à 9 jours d'incubation. Il est toutefois possible, qu'à ce stade, la sécrétion hormonale d'une médullaire femelle soit insuffisante pour arrêter la prolifération de cordons médullaires mâles. Des expériences sont en cours dans lesquelles nous éprouvons l'action d'une hormone sexuelle cristallisée.

ASSOCIATIONS D'ÉPITHÉLIUM GERMINATIF INDIFFÉRENCIÉ ET DE MÉDULLAIRE MÂLE

Les 43 associations réalisées dans cette série expérimentale ont donné les combinaisons suivantes: 26 couples épithélium germinatif mâle / médullaire mâle et 17 couples épithélium germinatif femelle / médullaire mâle.

Évolution morphologique des associations

L'épithélium germinatif indifférencié est associé à de la médullaire mâle gauche ou à du testicule droit, prélevé sur des embryons après le stade de la différenciation sexuelle. Dans ce type d'associations, on ne voit presque jamais l'épithélium germinatif, quel que soit son sexe, se différencier au sein de la médullaire qui l'entoure. Le testicule, découpé en plusieurs fragments que l'on place autour de l'épithélium germinatif, reforme un organe unique. En se fusionnant, les fragments enrobent complètement l'épithélium germinatif. Celui-ci ne se distingue plus de la masse devenue homogène et translucide.

Structure histologique des associations

Médullaire mâle

La médullaire mâle présente la structure caractéristique du testicule. C'est un ensemble dense et homogène de cordons repliés sur eux-mêmes. Les cordons testiculaires de Poulet se distinguent de ceux du Canard par leur calibre plus réduit.

Épithélium germinatif

Épithélium mâle. Nous avons d'abord associé l'épithélium germinatif génétiquement mâle de 6½ à 7 jours à du testicule de Canard (Tableau 3). Dans 5 cas sur 8, l'épithélium n'a pas été retrouvé. S'est-il différencié en cordons testiculaires qui se sont confondus avec ceux de la médullaire mâle associée, ou bien a-t-il périclité? Pour répondre à cette question, nous avons remplacé la médullaire mâle de Canard par de la médullaire mâle de Poulet. Dans 8 cas sur 18, l'épithélium germinatif s'est différencié en cordons que l'on distingue bien de

TABLEAU 3

Associations de médullaire testiculaire de Canard et de Poulet (9 à 11 jours d'incubation) et d'épithélium germinatif mâle de Canard (5 à 7 jours d'incubation)

Âge de l'épithélium germinatif en jours	*Nombre d'explants*	*Différenciation*				*Épithélium disparu*	*Nécrose*	*Médullaire associée*
		Épithélium germinatif *	*Cordons testiculaires*	*Cordons médullaires*	*Indéfinissable*			
6½ – 7	8	1	—	—	2	5	—	Canard
6½ – 7	9	1	3	—	—	2	3	Poulet
5 – 6	9	1	1	4	1	—	2	Poulet

* L'épithélium germinatif a gardé la structure qu'il présentait au moment de l'explantation.

ceux du Poulet (Planche 1, fig. 4). L'épithélium germinatif de 6½ à 7 jours a donné naissance à des cordons testiculaires (3 cas), celui de 5 à 6 jours à des cordons médullaires (5 cas, voir p. 418, note 1). Étant donné, que l'épithélium germinatif de cet âge ne se développe pas au contact de mésonéphros (Tableau 2*b*), on peut penser que la médullaire testiculaire exerce une action favorable sur la formation, voire la différenciation de cordons mâles.

TABLEAU 4

Associations de médullaire testiculaire de Canard et de Poulet (9 à 11 jours d'incubation) et d'épithélium germinatif femelle de Canard (5 à 7 jours d'incubation)

Âge de l'épithélium germinatif en jours	*Nombre d'explants*	*Différenciation*		*Épithélium disparu ou nécrosé*
		Cortex ovarien	*Indéfinissable*	
6½ – 7	7	4	2	1
5 – 6	10	6	2	2

Épithélium germinatif femelle. La culture au sein d'une médullaire mâle n'entrave pas la différenciation de l'épithélium germinatif femelle en cortex ovarien (Tableau 4; Planche 1, fig. 3). L'épithélium femelle de 5 jours donne naissance aux mêmes formations corticales, qu'il soit associé à de la médullaire femelle, à du mésonéphros ou à du testicule. Ce résultat apporte la preuve que

l'orientation de la différenciation femelle de l'épithélium germinatif est déterminée à 5 jours d'incubation, et qu'on ne peut plus, à ce stade, influencer son développement normal.

CONCLUSIONS GÉNÉRALES

La culture *in vitro* d'épithélium germinatif indifférencié en présence de médullaire testiculaire, de médullaire ovarienne ou de mésonéphros, a montré que l'épithélium germinatif est orienté vers la différenciation mâle et femelle à un stade précoce du développement embryonnaire. Ce stade est antérieur à celui où se manifeste la différenciation sexuelle dans l'embryon, mais il n'est pas le même dans les deux sexes.

La détermination de l'épithélium germinatif femelle est irrévocable dès 5 jours d'incubation, celle de l'épithélium mâle n'a lieu qu'à partir de 6 jours. De plus cette dernière est labile. On peut orienter la différenciation de l'épithélium mâle dans une direction opposée à la normale, jusqu'à environ 7 jours d'incubation. Au-delà de ce stade, on ne peut plus agir que partiellement sur sa différenciation normale.

Lorsque l'on cultive l'épithélium germinatif mâle de 5 à 6 jours d'incubation au contact de tissu neutre tel que le mésonéphros, il ne se développe pas. Par contre, si on l'associe à une médullaire, il se différencie suivant le sexe de celle-ci. Ainsi, en présence d'une médullaire mâle, il forme des cordons testiculaires; en présence d'une médullaire femelle, il évolue en cortex ovarien. Il n'est donc pas douteux que la médullaire est nécessaire, aux jeunes stades du développement, pour assurer la différenciation de l'épithélium germinatif mâle.

Il serait intéressant de rechercher le stade auquel l'épithélium germinatif femelle n'est pas encore orienté vers sa différenciation. Jusqu'à présent, il ne nous a pas été possible d'isoler l'épithélium germinatif de gonades de 4 jours d'incubation, mais d'autres expériences sont en cours qui permettront peut-être de résoudre cette question.

La féminisation de l'épithélium germinatif mâle, sous l'influence de la médullaire ovarienne, apporte une preuve supplémentaire de l'action féminisante du composant médullaire des gonades femelles de l'embryon d'Oiseau, qui a déjà été démontrée *in vivo* et *in vitro* par différents auteurs (Wolff, 1947; Wolff & Wolff, 1949, 1951; Wolff & Haffen, 1952; Mintz & Wolff, 1952, 1954).

RÉSUMÉ

1. L'épithélium germinatif indifférencié, isolé à la trypsine, de gonades embryonnaires de Canard âgées de 5 à 7 jours d'incubation, a été associé, en culture *in vitro*, à de la médullaire ovarienne et à de la médullaire testiculaire de gonades, prélevées après le stade de la différenciation sexuelle.

2. L'épithélium germinatif femelle se développe et se différencie en cortex ovarien dès l'âge de 5 jours, quelle que soit l'origine de la médullaire à laquelle

on l'associe (Planche 1, fig. 3; Planche 2, figs. 5, 6 *a*, *b*, *c*). Il suit la même évolution en présence de mésonéphros utilisé comme témoin (Planche 2, fig. 5).

3. L'épithélium germinatif mâle de 5 à 7 jours subit l'influence du tissu avec lequel il est en contact. En présence de médullaire testiculaire, il donne naissance à des cordons médullaires mâles (Planche 1, fig. 4). En présence de médullaire ovarienne, il se différencie en cortex ovarien (Planche 3, figs. 7 *a*, *b*, *c*). En présence de mésonéphros, il ne donne aucune différenciation. Ces résultats sont particulièrement démonstratifs dans le cas d'épithéliums germinatifs, prélevés entre 5 et 6 jours d'incubation.

SUMMARY

1. Undifferentiated germinal epithelium was isolated following trypsinization from embryonic duck gonads of 5 to 7 days of incubation. This epithelium was associated with ovarian and testicular medullae taken from sexually differentiated embryos.

2. Ovarian cortex differentiates from female germinal epithelium of 5 days, whatever the source of the medullary tissue with which it is associated (Plate 1, fig. 3; Plate 2, figs. 5, 6 *a*, *b*, *c*). In control experiments the female epithelium undergoes similar development in the presence of mesonephros (Plate 2, fig. 5).

3. Five- to seven-day male germinal epithelium is influenced by tissues with which it is in contact. In the presence of testicular medulla it gives rise to male medullary cords (Plate 1, fig. 4). In the presence of ovarian medulla, ovarian cortex differentiates (Plate 3, figs. 7 *a*, *b*, *c*). When in contact with mesonephros no differentiation of the male epithelium ensues. These results are particularly clear in the case of germinal epithelia explanted between 5 and 6 days of incubation.

TRAVAUX CITÉS

Kofcke, H. U. (1958). Normalstadien der Embryonalentwicklung bei der Hausente (*Anas Boschas Domestica*). *Embryologia*, **4**, 55–78.

Mintz, B., & Wolff, Ét. (1952). Sur les greffes coelomiques de la médullaire ovarienne d'embryons de Poulet: l'évolution des greffons et leur action féminisante sur les hôtes mâles. *C.R. Soc. Biol. Paris*, **146**, 494–5.

—— —— (1954). The development of embryonic chick ovarian medulla and its feminizing action in intracoelomic grafts. *J. exp. Zool.* **126**, 511–36.

Moscona, A. (1952). Cell suspensions from organ rudiments of chick embryos. *Exp. Cell Res.* **3**, 535–9.

Wolff, Ét. (1947). Recherche sur l'intersexualité expérimentale produite par la méthode des greffes de gonades à l'embryon de Poulet. *Arch. Anat. micr. Morph. exp.* **36**, 69–90.

—— & Haffen, K. (1952*a*). Sur une méthode de culture d'organes embryonnaires *in vitro*. *Texas Rep. Biol. Med.* **10**, 463–72.

—— —— (1952*b*). Sur le développement et la différenciation sexuelle des gonades embryonnaires d'Oiseau, en culture *in vitro*. *J. exp. Zool.* **119**, 381–404.

—— —— (1952*c*). Action féminisante de la gonade droite de l'embryon de Canard en culture *in vitro*. *C.R. Soc. Biol. Paris*, **146**, 1772–4.

—— —— (1952*d*). Sur la différenciation sexuelle des gonades embryonnaires de l'embryon de Poulet en culture *in vitro*. *Ann. Endocr.* **13**, 724–31.

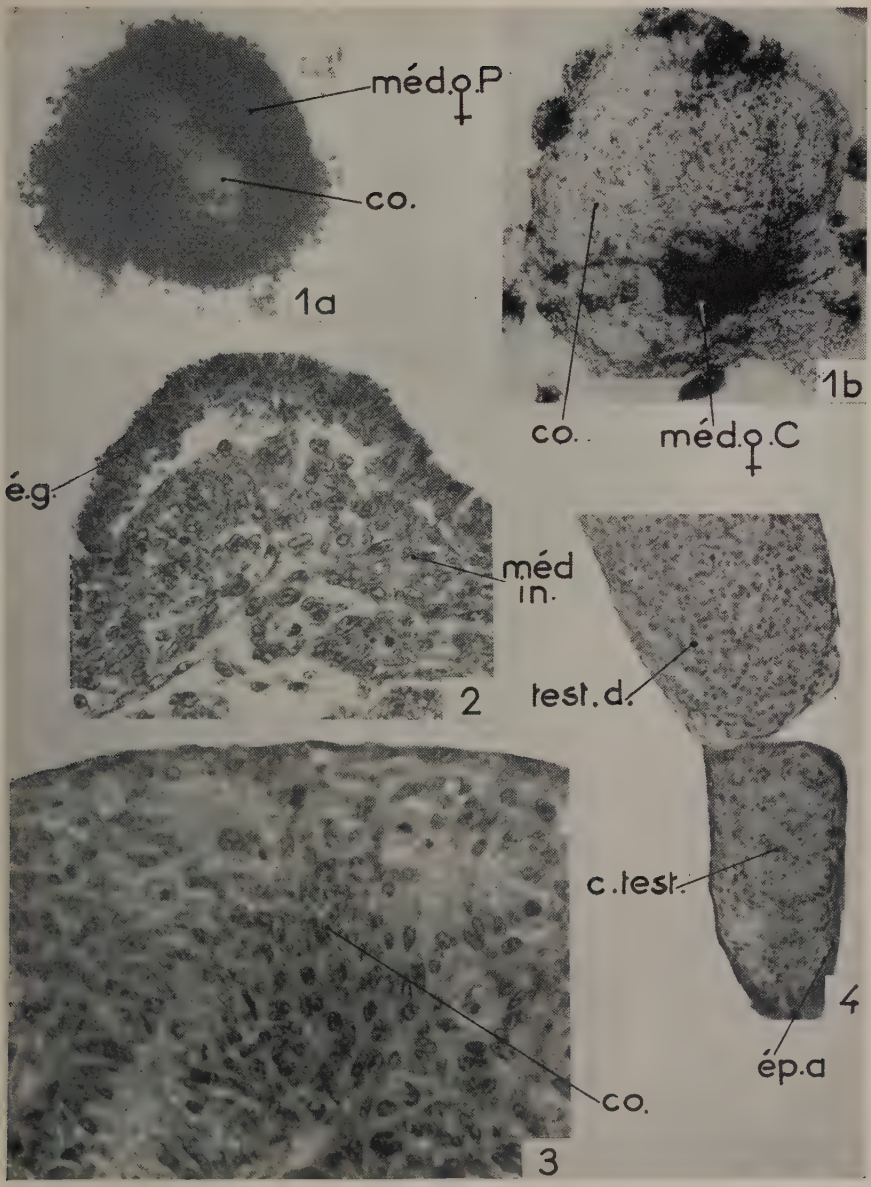

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Planche 1

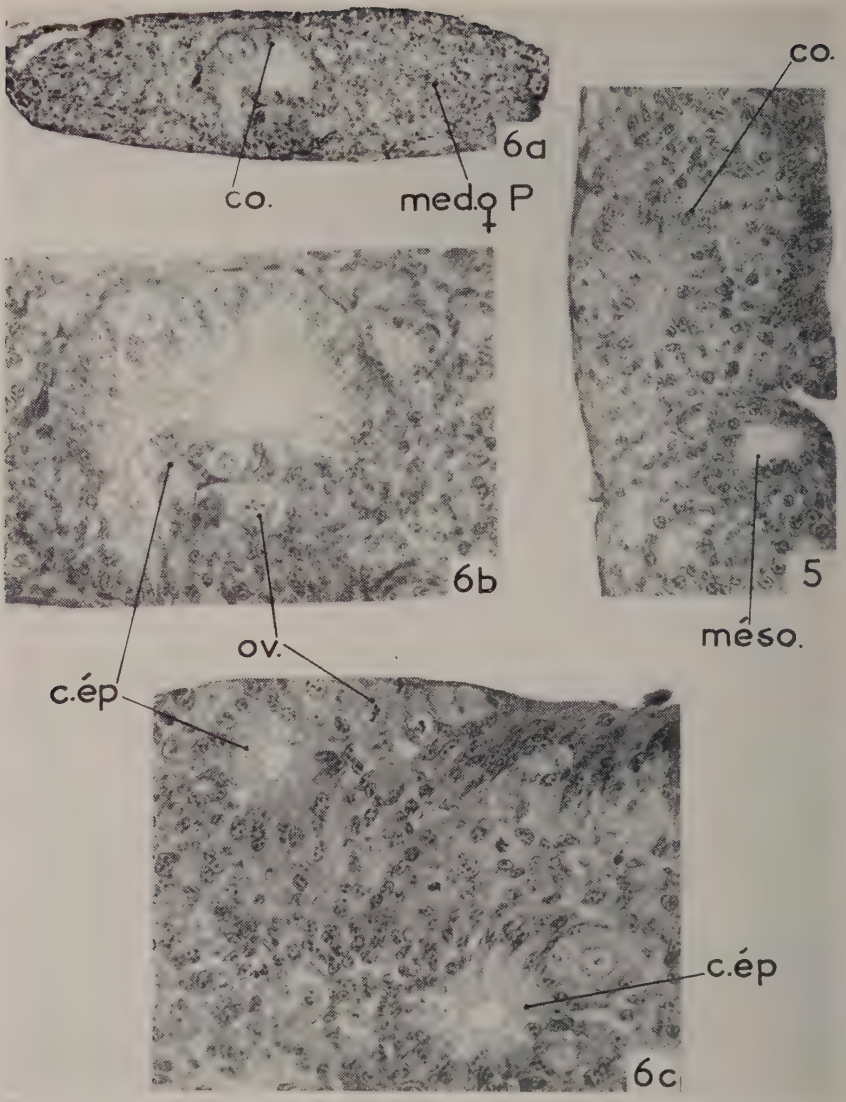

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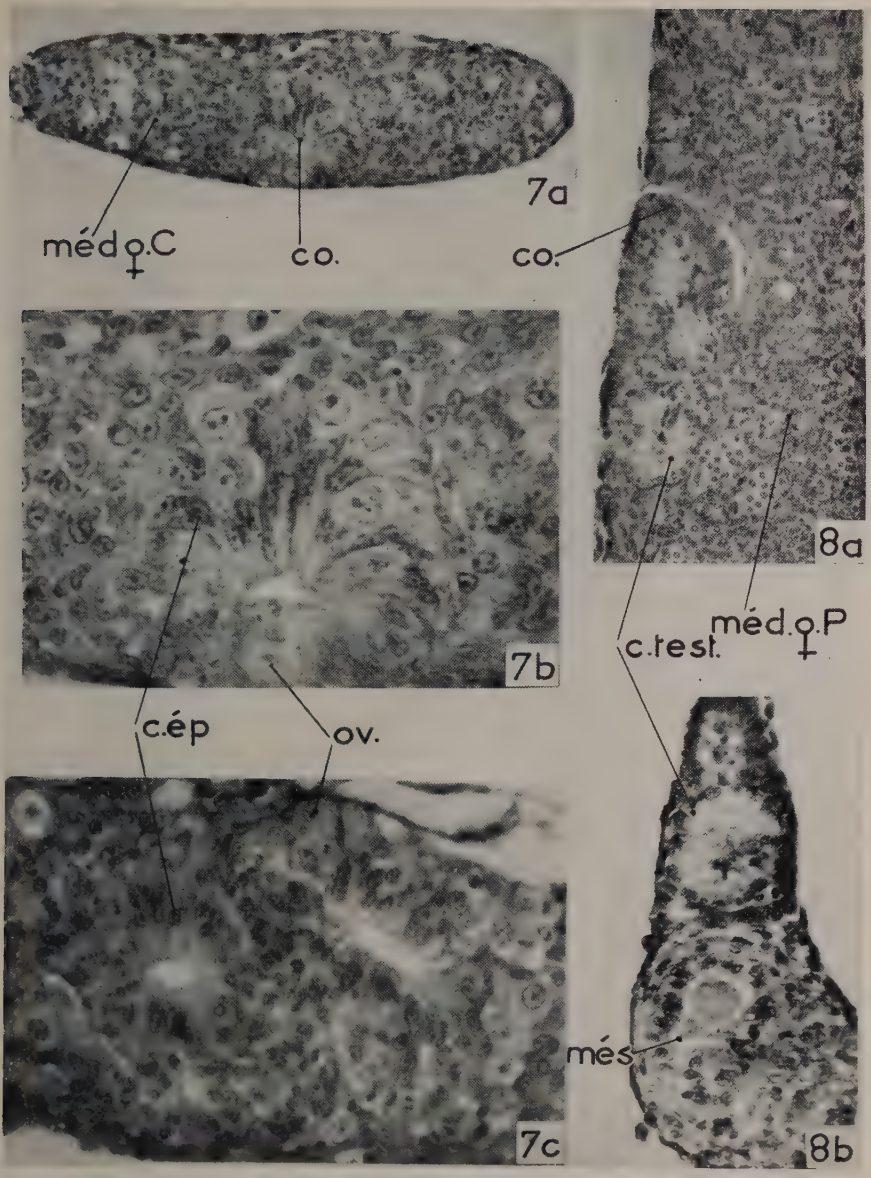
Planche 2



K. HAFFEN

Planche 3

Wolff, Ét., & Haffen, K. (1959). La culture *in vitro* de l'épithélium germinatif isolé des gonades mâles et femelles de l'embryon de Canard. *Arch. Anat. micr. Morph. exp.* **48**, 331–45.

—— & Wolff, Em. (1949). Application de la méthode de castration à l'embryon de Canard: sur deux tests de l'activité précoce des gonades embryonnaires, la syrinx et le tubercule génital. *C.R. Soc. Biol. Paris*, **143**, 529–31.

—— —— (1951*a*). The effects of castration on bird embryos. *J. exp. Zool.* **116**, 59–97.

—— —— (1951*b*). Mise en évidence d'une action féminisante de la gonade droite chez l'embryon femelle des Oiseaux, par les expériences d'hémicastration. *C.R. Soc. Biol. Paris*, **145**, 1218–19.

EXPLICATION DES PLANCHES

Planche 1

Fig. 1. Associations d'épithélium germinatif de 6 jours et de médullaire ovarienne de 11 jours d'incubation, photographiées après 5 jours de culture. *co.*, cortex; *méd.* ♀ *P* et *méd.* ♀ *C*, médullaires femelles de Poulet et de Canard.

a, épithélium germinatif ♂ développé en cortex au contact d'une médullaire ♀ de Poulet. La médullaire ♀ de Poulet, non régressée, est opaque et granuleuse. Elle entoure l'épithélium germinatif devenu translucide. ×44.

b, épithélium germinatif ♀ développé en cortex au contact d'une médullaire ♀ de Canard. Cette dernière, considérablement régressée, n'est plus représentée que par quelques petites plages granuleuses. ×69.

Fig. 2. Coupe transversale à travers une gonade indifférenciée de 6 jours d'incubation. Séparation de l'épithélium germinatif et de la médullaire par l'action de la trypsine diluée. *é.g.*, épithélium germinatif; *méd. in.*, médullaire indifférenciée. ×420.

Fig. 3. Détail d'un épithélium germinatif ♀ de 6 jours cultivé au contact d'une médullaire testiculaire de 10 jours d'incubation. Il s'est différencié en cortex ovarien dont la structure est identique à celle d'un épithélium germinatif différencié au contact de mésonéphros ou au contact de médullaire ♀. Durée de culture: 5 jours. *co.*, cortex. ×490.

Fig. 4. Association d'épithélium germinatif ♂ de 6 jours et de testicule droit de Poulet de 9 jours d'incubation. L'épithélium germinatif, en bas, a donné naissance à des cordons testiculaires, qu'entoure un épithélium aplati. Durée de culture: 6 jours. *test. d.*, testicule droit associé; *ép. a.*, épithélium aplati; *c. test.*, cordons testiculaires. ×169.

Planche 2

Fig. 5. Aspect de l'épithélium germinatif ♀ de 5½ jours d'incubation, cultivé pendant 4 jours au contact de mésonéphros. Il s'est différencié en cortex, étalé à la périphérie, replié sur lui-même au centre. *co.*, cortex; *méso.*, mésonéphros. ×169.

Fig. 6. Associations d'épithélium germinatif ♀ de 5½ jours et de médullaire ovarienne de Poulet de 10 jours d'incubation. Durée de culture: 5 jours. *méd.* ♀ *P*, médullaire femelle de Poulet; *co.*, cortex; *c. ép.*, cordons épithéliaux; *ov.*, ovogonies.

a, vue d'ensemble montrant la fusion intime entre l'épithélium germinatif ♀ différencié en cortex et la médullaire ♀ de Poulet. Cette dernière est formée de petits cordons plus ou moins testiculaires, serrés les uns contre les autres. ×150.

b, *c*, détails du cortex ovarien différencié à partir de l'épithélium germinatif ♀. Noter la formation de cordons épithéliaux; ils comportent plusieurs assises de hautes cellules étroites et prismatiques. Des ovogonies sont intercalées entre les cellules épithéliales ou groupées en îlots à la base des cordons. ×420.

Planche 3

Fig. 7. Associations d'épithélium germinatif ♂ de 6 jours et de médullaire ovarienne de Canard de 11 jours d'incubation. Durée de culture: 5 jours. *méd.* ♀ *C*, médullaire femelle de Canard; *co.*, cortex; *c. ép.*, cordons épithéliaux; *ov.*, ovogonies.

a, vue d'ensemble d'une association. L'épithélium germinatif ♂ s'est différencié en cortex ovarien dont la structure est identique à celle d'un cortex différencié à partir d'un épithélium germinatif ♀. La médullaire ♀ de Canard qui l'entoure est en voie de régression. ×150.

b, *c*, détails montrant la structure du cortex différencié à partir d'un épithélium germinatif ♂. Sa structure est comparable à celle d'un cortex ovarien. Noter la formation de cordons épithéliaux et la présence d'ovogonies. Comparer ces figures avec les figures 6 *a*, *b*, *c*. ×378 et 420.

FIG. 8. Explantation de moitiés d'épithélium germinatif ♂ de 9 jours d'incubation. *co.*, cortex; *c. test.*, cordons testiculaires; *méd.* ♀ *P*, médullaire femelle de Poulet; *més.*, mésonéphros.

a, moitié cultivée pendant 5 jours en présence de médullaire ♀ de Poulet de 10 jours d'incubation. Noter la différenciation simultanée de cortex et de cordons testiculaires. ×200.

b, moitié cultivée pendant 5 jours en présence de mésonéphros. Persistance partielle de l'épithélium germinatif à l'état d'assise aplatie. Différenciation de cordons testiculaires. ×240.

(*Manuscript received 30:iv:60*)

The Influence of the Hypophysis and the Thyroid on the Ultimobranchial Body of the Anura of Israel

by DVORAH BOSCHWITZ[1]

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WITH ONE PLATE

Introduction

THE ultimobranchial body of *Hyla arborea* L., *Rana ridibunda* Pall., *Pelobates syriacus* Boettger, and *Bufo viridis* Laur., the common anurans of Israel, is a paired organ, situated on both sides of the aditus laryngis. Its development runs parallel to the fluctuating activity of the thyroid (Boschwitz, 1960).

(*a*) In the premetamorphic period of relative thyroid dormancy, the ultimobranchial body consists of one follicle with single-layered epithelium in *Hyla*, *Rana*, and *Bufo*, and of a coiled tube with parafollicular cells in *Pelobates*. A capsule with capillaries surrounds the organ.

(*b*) In the period of metamorphosis up to the beginning of tail-resorption, during which thyroidal activity is heightened, the epithelium of the ultimobranchial body becomes pseudostratified, and the size of the follicle enlarges. In *Pelobates* the coiled tube changes into several single-layered follicles with parafollicular cells. The whole organ becomes less dispersed. The follicles of all 4 species contain a small amount of faintly eosinophilic coagulum, and often normal-appearing nuclei are embedded therein.

(*c*) In the postmetamorphic period, during which thyroid activity declines, the ultimobranchial body enlarges and differentiates. There is one large follicle with pseudostratified epithelium and shallow folds in *Hyla*, and one or two large follicles with similar epithelium but deep folds in *Rana*. There are many small follicles with single-layered epithelium and interspersed clusters of parafollicular cells in *Bufo* and *Pelobates*, concentrated in an ovoid organ. The coagulum in the organ of all 4 species is eosinophilic, and includes nuclei, desquamated from the follicular wall.

Since the development of the ultimobranchial body parallels thyroid activity in these anurans and its postmetamorphic atrophy in *Xenopus laevis* may be influenced by the thyroid and the hypophysis (Sterba 1950; Saxén & Toivonen 1955), the influence of the hypophysis and thyroid on the ultimobranchial body has been experimentally investigated and the results are described in the

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present paper. A similar investigation of urodeles was performed by Steinitz & Stone (1954).

MATERIAL AND METHODS

Details of the various experiments are shown in Table 1.

The influence of the hypophysis in adults was investigated by means of hypophysectomy in *R. ridibunda* Pall., whose ultimobranchial body consists of follicles only, and in *B. viridis* Laur., which has parafollicular cells, too.

TABLE 1

Details of experiments. The three stages are: I, premetamorphic; II, metamorphic; and III, postmetamorphic

Experiment	*Species*	*Stage*	*Number of animals*	*Maximum duration of experiment (days)*
Hypophysectomy	*Rana*	III	20	111
	Bufo	III	7	25
Thiourea: 0·03%	*Hyla*	I	30	85
		II	30	
		III	20	
0·03%	*Rana*	I	20	60
		II	20	
0·06%	*Rana*	I	10	60
		II	10	
0·03%	*Bufo*	I	20	50
		II	20	
Thyroidectomy	*Rana*	III	6	65
	Bufo	I	10	30
		II	10	
Hypophysectomy + thyroidectomy	*Rana*	III	4	details in text
Thyroid powder (0·1 g./300 c.c. water)	*Bufo*	I	20	10
		II	20	

Under ether anaesthesia the animal was laid on its back and a median incision made in the mucosa of the roof of the mouth posterior to the eyes. When the resulting flaps were laid aside, the hypophysis became visible through the floor of the skull (parasphenoid). A square window was opened in the bone and the gland excised. Bone and flaps were then returned to their original position. After varying intervals the animals were killed. Serial sections of the region determined the degree of success of the operation. As controls, unoperated and sham-operated animals were used.

The influence of the thyroid, during its three periods of activity, was studied by means of experimental hypo- and hyper-thyroidism. Hypothyroidism was caused by solutions of thiourea of 0·3 per cent. in tadpoles of *H. arborea* L., *R. ridibunda* Pall. and in *B. viridis* Laur. Solutions of thiourea of 0·06 per cent. were used in *R. ridibunda* Pall. only. Since the goitrogen may influence the ultimobranchial body directly and not via the thyroid, thyroidectomy, too, was

performed in *R. ridibunda* Pall. and in *B. viridis* Laur. If the effect was similar to that of thiourea, it would indicate that thyroid hormone deficiency was responsible for the changes in the ultimobranchial body. Hyperthyroidism was produced by administration of thyroid powder in tadpoles of *B. viridis* Laur. Twenty limbless tadpoles and 20 with hind limbs were kept in tap-water containing 0·1 gm. thyroid powder per 300 c.c. during 5 to 10 days. They were then transferred into tap-water and kept alive therein for a further period of 5 to 10 days. If the effect on the ultimobranchial body is opposite to that of both forms of thyroid depletion, it may be assumed that the ultimobranchial body is affected by the extra thyroid hormone.

Two naturally athyreotic giant tadpoles of *P. syriacus* Boettger served for the study of the development of the ultimobranchial body in the complete absence of the thyroid.

As hypophysectomy produced the opposite effect to thyroidectomy, both operations were combined in four adults of *R. ridibunda* Pall.

Serial sections of experimental animals and controls were prepared. The whole region between the optic chiasma and the apex of the heart was cut; this included the hypophysis, the thyroid and the ultimobranchial body or any remnant of the former two after intended extirpation. Sections were 10 μ thick. Staining was by Ehrlich's haematoxylin and eosin.

EXPERIMENTAL RESULTS

Hypophysectomy in Rana *and* Bufo *adults*

Twenty specimens of *Rana* and 7 of *Bufo*, of an initial overall length of 3·0–3·5 cm., survived the operation. One *Rana* specimen was maintained 111 days postoperatively without, according to the histological series, any trace of an hypophysis. Its brain was undamaged. The ultimobranchial (Plate, fig. 5), instead of being ovoid, became elongated and flat, with a thin capsule and few capillaries. The epithelium was no longer folded or pseudostratified. It became simple and the cells lost cytoplasm and their crowded appearance. The nuclei became small or attenuated, and only occasionally protruded somewhat into the lumen. This became so narrow that the cells of one side almost touched those opposite; coagulum and nuclei were missing. The other animals were maintained for shorter periods, ranging from 17 to 63 days. The changes in the ultimobranchial body were similar but less pronounced: a coagulum with nuclei sometimes persisted (follicles lacking coagulum may also occur in unoperated animals). Sometimes the lumina showed narrow diverticula, probably relics of formerly distended branches, like those seen in the controls. In brief, the organ had unmistakably involuted to a more or less inactive state.

In three cases the organ was not very dissimilar to the controls, and histological examination showed that a portion of the pars anterior had remained intact. Hypophysial control over the organ may thus be quantitative. In these

cases, the ultimobranchial body on one side was more affected than on the other. An asymmetry of the remaining hypophysis was not observed.

In *Bufo* the capillary bed was unusually expanded, and the follicles atrophied by shrinking (Plate, figs. 1, 2). Occasionally, a slight amount of coagulum was retained in their lumina; but mostly they were flattened and empty. Hence, the follicles appeared cordlike and could be mistaken for clumps of parafollicular cells.

Hypothyroidism in Hyla, Rana, *and* Bufo

The ultimobranchial body of *Hyla* is exceptional among the four species studied, as it undergoes only slight changes during development. It may, therefore, be presumed that the influence of the thyroid on it is comparatively small. Consequently, experimental reduction of thyroid activity would not be expected to cause as much change as might occur in organs whose growth and differentiation is more conspicuously correlated with the physiological decline of thyroid secretion.

The ultimobranchial body of animals kept in 0·03 per cent. thiourea up to 80 days remained very similar to controls. Only one tadpole and two adults survived for 85 days. When killed, the ultimobranchial body was larger than that of the controls, and its epithelial surface was increased by low folds bulging into the enlarged lumen. The organ resembled that of an older animal.

The development of the ultimobranchial body of *Rana* would seem to augur a relatively more sensitive response to the removal of thyroid influence than in the case of *Hyla*. This was found to be true. The histological changes of the ultimobranchial body of animals kept in 0·03 per cent. or 0·06 per cent. thiourea solution for 30 to 60 days were different if the experiments were performed during the three different periods mentioned in the introduction:

Group A: Tadpoles up to the stage before precartilage develops in the internal forelimb buds; a period of low thyroid activity.

Group B: Tadpoles older than those of group A, up to the beginning of tail-resorption; a period of high thyroid activity.

Group C: Tadpoles older than those of group B; a period of relative thyroidal decline.

In group A, only the lumen of the ultimobranchial body was enlarged. The epithelium remained cuboidal as in the controls: accordingly, the thyroid inhibitor elicited only a weak effect.

In group B, after 30 days in 0·03 per cent. thiourea the unifollicular ultimobranchial body and its lumen were considerably enlarged. Moreover, in two cases a second follicle appeared on each side. After the same length of time in 0·06 per cent. thiourea or 40 days in 0·03 per cent., the organ became enlarged, reaching at least double the control diameter. After 40 days in 0·06 per cent. solution, the organ hypertrophied to almost three times the control diameter. The epithelium in these cases became densely packed and columnar, and

numerous cells protruded into the lumen. The coagulum, however, failed to increase. The effect of thiourea was therefore more marked the stronger the solution and the longer the exposure.

In group C, the ultimobranchial body showed an increase in the epithelial surface area by means of folds protruding into the lumen. Capillaries from the capsule accompanied the epithelium into the folds, but there was no increase in size of the organ.

Thyroidectomy was performed in *Rana* adults only. Where the operation was completely successful, again an increase of epithelial surface area of the ultimobranchial body without recognizable hypertrophy of volume was seen. The epithelium was simple columnar or pseudostratified and distended capillaries invaded the folds protruding into the lumen (Plate, fig. 4). The nuclei were lengthened and surrounded by a large amount of eosinophilic cytoplasm, many bulging into the lumen and some almost completely detached from the epithelial wall. The organ seemed to be in a state of heightened activity on the basis of its increased surface area and the absence of pycnosis, in contrast to its condition in hypophysectomized animals. The lumen was branched and contained an eosinophilic coagulum and many nuclei. If no folds of epithelium were present, the lumen was considerably distended.

The appearance of heightened activity of the ultimobranchial body was less marked where the thyroidectomy was less successful; but the degree of activity could not be correlated with the size of the remnant or the elapsed time: there may have been a period of dormancy of unknown duration following injury of the thyroid follicles or of their blood-supply, after which limited recovery may occur.

The results of thyroidectomy accorded well with those of thiourea treatment.

As the response of the *Bufo* ultimobranchial body to experimental hypothyroidism is like that in *Rana*, the same classification is used.

Group A: Tadpoles about 3 cm. long, without limb-buds, in contrast to controls showed no indication of entering metamorphosis even after 48 days in thiourea solution. Their ultimobranchial body of *c.* 65 μ diameter had a slight elongation, but the lumen was of normal size. The same effect resulted from thyroidectomy after only 30 days if the extirpation was performed on tadpoles of the same group.

Group B: Tadpoles with limb-buds kept in thiourea solution continued to develop at a slow rate. Their ultimobranchial body displayed a marked hypertrophy, and, in one case, multifollicularity. The unusual diameter of 140 μ and the enlarged lumen of the follicle, containing increased coagulum and a number of nuclei, is characteristic of the normal toad after metamorphosis. One tadpole, with 1-cm. hind limbs at the beginning of the experiment, was killed after 50 days: an extra pair of large follicles had developed from the original primordium, and these were situated a considerable distance away from, and without connective-tissue links to, the follicle. While the latter had a normal diameter of

70 μ, the additional follicle on one side was almost twice normal size and that on the other side almost three times normal size. Their lumen was enlarged and contained abundant coagulum.

Complete thyroidectomy performed on tadpoles of group B proved more efficacious than thiourea, producing a larger organ and as many as three somewhat smaller follicles with relatively large lumina on each side in extreme cases (Plate, fig. 3). Other tadpoles, only partially thyroidectomized, displayed transitional responses ranging from a somewhat elongated follicle on both sides to 2 follicles on one side and 1 on the other, 2 follicles on each side, and 3 follicles on one side and 2 on the other. In all cases, a common and extremely thin capsule containing wide capillaries and melanophores surrounded the follicles. In two cases the additional follicle was very small, and appeared some distance away, outside the capsule, and closely applied to the epithelium of the gill arch as in the primordium stage. Its structure, however, characterized it as part of the ultimobranchial body. Again, the changes were not proportional to the time elapsed or to the size of the fragments of the thyroid left after the operation.

Only once has a supernumerary follicle been observed in a normal tadpole, due, perhaps, to a malfunctioning thyroid.

Group C: Four-limbed tadpoles, which had completed metamorphosis, were killed after 30 days in thiourea solution. The volume of the ultimobranchial body was normal, but the surface area of its wall was increased by folds, which protrude into the lumen. Parafollicular cells typical of normal *Bufo* adults were not discerned.

Thyroidless giant tadpoles of P. syriacus

The normal *P. syriacus* tadpole attains a size of 110 mm. before the appearance of hind limbs. In the spring of 1956, however, some 30 *P. syriacus* tadpoles of 175-mm. size and still lacking limbs were found in rain-water ponds near the Israel coastal town of Holon (Grid Ref. 1295/1953). Most were kept in aquarium tanks for 9 months. Only three of them produced limbs of minute size.

Giant tadpoles of *P. fuscus* (Mertens, 1947) are known from cold Alpine lakes, where it is assumed that, owing to the coldness of the water, they continued to grow until metamorphosing in the second year of life. Cold water could not be the cause of gigantism in the Israel tadpoles, as the ponds concerned have a noon temperature of 30° C. They dry up during the summer, and only individuals metamorphosed by then could possibly survive. Serial sections of the entire thyroid region (plus an adequate amount of nearby tissue) of two tadpoles were examined. The thyroid gland was completely lacking (Boschwitz, 1957). Tadpoles of *R. pipiens* deprived of the thyroid anlage are known to grow to unusual dimensions (Allen, 1918) without ever developing limb buds.

The ultimobranchial body of the giant *Pelobates* tadpoles displayed the typical response to lack of thyroid. It was twice as large as in controls; having progressed from the coiled tube stage, it assumed the ovoid form of later

metamorphic stages and it was therefore compared with the ultimobranchial body of animals of this stage. Many follicles were more voluminous, as was also the quantity of coagulum. The number of follicles and of parafollicular cells was greater than normal. But the embryonic feature of close association with the epithelium of origin was still retained.

The effect on the ultimobranchial body of hyperthyroidism induced by thyroid powder in Bufo *tadpoles*

The thyroid powder and thiourea effects could be distinguished early by the amount of faeces. Animals treated with thiourea produced more than the controls, while those treated with thyroid powder produced almost none at all. The latter, owing presumably to accelerated metamorphosis, began to live exclusively on their own tissue (for instance the tail).

A number of animals already displayed forelimbs two days after the 5-day thyroid powder treatment. Their ultimobranchial body measured then only 40–60 μ in diameter, instead of the normal 100–120 μ. It retained the embryonic spherical shape, clinging to the branchial arch epithelium, as if but recently budded off. The capsule was meagre, with few capillaries. The epithelium had the usual pseudostratification, but the nuclei were neither crowded together nor protruded into the lumen, which only occasionally contained a little coagulum and a nucleus or two. The extreme under-development indicated that morphogenesis had been halted or had regressed, although metamorphosis progressed.

Animals killed 5 and 10 days after the treatment showed more pronounced degree of shrinking of the ultimobranchial body. The epithelium pushed so many folds into the lumen that the cavity was almost choked, as a result not of hyperplasia but of contraction, as in hypophysectomized *Rana* adults. The cytoplasm was shrunken around pycnotic nuclei. The connective tissue did not accompany the epithelial folds. Melanophores ringed the capsule. A comparison with controls led to the conclusion that these melanophores originally lay against the follicle but that, as the epithelium shrank away and the capsule dwindled, a detached layer of melanophores appeared.

Hypophysectomy and thyroidectomy in adult Rana

Since hypophysectomy and thyroidectomy were found to have opposite effects on the ultimobranchial body, it was of interest to carry out both operations in one animal. The effect of subsequent thyroidectomy was expected to reduce the involution produced by an initial hypophysectomy.

Four *Rana* adults were hypophysectomized, and the thyroid excised 40, 42, 48, and 54 days later, respectively. The first two animals were killed three days, the third 10 days, and the fourth 15 days after the second operation. Even the first two animals showed a renewed proliferation of cells and expansion of the lumen as compared with the involution subsequent to hypophysectomy alone.

The blood supply had also increased, but the capillaries did not yet protrude into the folds as in animals only thyroidectomized. The other two animals revealed an increasing number of folds and an eosinophilic coagulum including some cells (Plate, fig. 6).

DISCUSSION

Although none of the foregoing results provides a clue to the function of the ultimobranchial body, they suggest that its activity is somehow associated with that of the hypophysis and the thyroid. It is possible to formulate the following tentative generalizations: the hypophysis maintains the ultimobranchial body, since hypophysectomy causes its atrophy; and the thyroid inhibits the organ, which hypertrophies in hypothyroidism. The normal ultimobranchial body may result from a combined effect of antagonistic factors from both glands.

The experiments were performed during different developmental periods, and the results confirmed that the fluctuating activity of the thyroid is not merely coincidental with the changes that take place in the ultimobranchial body during development.

Normally, the ultimobranchial body develops after the rudiment of the thyroid has appeared, but, as seen in the congenitally athyreotic giant tadpoles of *P. syriacus*, this sequence is temporal rather than causal. The growth of their ultimobranchial body was unusual, as it was never interfered with by the inhibitory thyroid hormone (Eggert, 1938; Schaefer, 1938). The ultimobranchial body of human athyreotic neonati, likewise, fails to atrophy (Getzowa, 1911).

Until the appearance of precartilage in the forelimb buds, the inhibition of the ultimobranchial body growth by the thyroid (up to this stage functionally dormant) is negligible, as indicated by its slight expansion after thyroid deprival. A significant response seems not yet possible, confirming that the ultimobranchial body is dependent on the quantity of thyroid hormone circulating in the blood. The increase in thyroid activity at metamorphosis diminishes the growth rate of the ultimobranchial body. The degree of this interference may be measured by the increase of surface area in the ultimobranchial body of tadpoles subsequent to experimental hypothyroidism, produced either by thiourea or by thyroidectomy.

The multifollicular state is connected with low thyroid activity, either during the first developmental stage of the thyroid, when the pharynx epithelium is able to create new follicles, or in the adult (*Bufo*) with the physiological decline of the thyroid. It seems reasonable, therefore, to explain the multifollicularity of the operated animals as caused by the deprivation of thyroid secretion. When the single-layered epithelium of the follicle transmutes into a pseudostratified one, the potency of the pharynx epithelium is gradually lost, possibly owing to the inhibitory thyroid hormone, now secreted in increasing amount.

After thyroid deprivation at this time, the single follicle hypertrophies and becomes some two or three times as large as the normal. This increase in secreting surface is enhanced by folds, protruding into the lumen. Follicular cells and coagulum display the features of activity (*Hyla*, *Rana*, *Bufo*). The later the reduction of thyroidal influence the less the added growth attained by the ultimobranchial body. Parafollicular cells (*Bufo*) never appear before the end of metamorphosis. Apparently, their development depends on factors available only in adults. No obvious changes were found in parafollicular cells in any of the experiments.

Addition of thyroid hormone during metamorphosis supplements the intensive activity of the thyroid. The growth rate of the ultimobranchial body is halted, as evidenced by its shrunken appearance and poor blood-supply. In the post-metamorphic period, experimental repression of thyroid activity reinforces the natural spurt of development of the ultimobranchial body due to the physiological decline of thyroid secretion.

Since thyroidectomy and thiourea treatment have precisely the same effect on the ultimobranchial body, and thyroid powder exactly the opposite, it may be concluded that these substances influence the organ in the same way as the fluctuating secretion of the thyroid. Another explanation of the thyroid–ultimobranchial body relationship could be that after anti-thyroid treatment or after thyroidectomy the TSH-secretion of the pituitary is stimulated and causes the hypertrophy of the ultimobranchial body.

Of course, it is not certain that the hypertrophy of the ultimobranchial body is indicative of its heightened activity, since we know that hypertrophy of the thyroid may be connected with hypofunction as well as with hyperfunction. Additional experiments are needed to confirm that hypertrophy of the ultimobranchial body is a symptom of its heightened secretion.

As expected, it was found that hypophysectomy of adults has the opposite effect to that of thyroidectomy. A combination of both extirpations, i.e. thyroidectomy some days after hypophysectomy, displayed a combination of effects.

Special explanation is needed for the ultimobranchial body of the one frog which survived hypophysectomy for 111 days: here, too the combined effect was expected, as after so long a period the secretion of thyroxine is diminished in consequence of the missing thyreotropic hormone. But the ultimobranchial body atrophied without any sign of renewed activity. A possible explanation seems to be that the ultimobranchial body is controlled by the thyroid gland from the time of appearance of the hind-limb buds until the end of metamorphosis. It reacts to the fluctuations of thyroid secretion as described. In the adult a direct influence of the hypophysis becomes predominant and therefore the involution of the ultimobranchial body after hypophysectomy progresses steadily (Eggert, 1938; Schaefer, 1938). Of course, hypophysectomy in tadpoles is necessary to confirm this assumption. Yet another explanation is possible: if the hypertrophy of the ultimobranchial body after thyroidal decline is caused

by the heightened TSH-secretion of the pituitary, its extirpation would produce an atrophied ultimobranchial body. Extirpation of both hypophysis and thyroid, with subsequent administration of thyroid powder and/or TSH, would contribute to the solution of this problem. Experiments with thyroxine administration after hypophysectomy are needed to differentiate between the influence of the hypophysis and the thyroid on the ultimobranchial body after metamorphosis.

The structure and the accelerated growth of the ultimobranchial body when the thyroid secretion declines, and its involution effected by rising amounts of thyroid hormone, are proof that the ultimobranchial body has a secretion which is not comparable to that of the thyroid. Hence the assumption (summarized by Lynn & Wachowsky, 1951), that the ultimobranchial body is a 'lateral thyroid', is not acceptable. Another differential feature of the ultimobranchial body is its inability to accumulate I^{131}, as shown in the mouse (Gorbman, 1947) and confirmed for *Xenopus laevis* by Saxén & Toivonen (1955).

SUMMARY

1. The ultimobranchial body originates independently of the thyroid, as seen in congenitally athyreotic tadpoles of *P. syriacus*.

2. Experiments concerning the influence of the hypophysis and thyroid on the ultimobranchial body were performed on *H. arborea*, *R. ridibunda*, and *B. viridis*.

3. With the beginning of metamorphosis, the increasing thyroid secretion inhibits the original growth rate of the ultimobranchial body; experimental hyperthyroidism enhances this physiological interference, so that the follicle shrinks.

4. Experimental hypothyroidism causes hypertrophy of the ultimobranchial body, evidenced by an increase in surface area of the follicle. This effect is negligible before metamorphosis, significant during metamorphosis, and less extreme in young adults, when thyroid activity declines.

5. Hypophysectomy in young adults causes atrophy of the organ.

6. It is assumed that the hypophysis maintains the ultimobranchial body and the thyroid inhibits its growth and activity. The antagonistic effects of both glands produce the normal state of the organ.

RÉSUMÉ

Influence de l'hypophyse et de la thyroïde sur le corps ultimobranchial des Anoures d'Israël

1. L'origine du corps ultimobranchial est indépendante de celle de la thyroïde, comme l'indiquent certains têtards de *Pelobates syriacus* frappés d'agénésie congénitale de la thyroïde.

2. Des expériences qui montrent le rôle de l'hypophyse et de la thyroïde sur

le corps ultimobranchial ont été faites sur *Hyla arborea*, *Rana ridibunda*, et *Bufo viridis*.

3. Au début de la métamorphose, l'augmentation de la sécrétion thyroïdienne entraîne une diminution du taux de croissance du corps ultimobranchial. L'hyperthyroïdisme expérimental exalte cette inhibition et détermine l'étrécissement du follicule.

4. L'hypothyroïdisme expérimental amène l'hypertrophie du corps ultimobranchial, révélée par l'augmentation de la surface du follicule. Cette action est négligeable avant la métamorphose, significative pendant cette période et moins importante chez les jeunes adultes dont l'activité thyroïdienne diminue.

5. L'hypophysectomie des jeunes adultes provoque l'atrophie du corps ultimobranchial.

6. On en conclut que l'hypophyse maintient le corps ultimobranchial et que la thyroïde inhibe sa croissance et son fonctionnement. L'état normal de cet organe résulte de l'effet antagoniste de ces deux glandes.

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REFERENCES

Allen, B. M. (1918). Results of thyroid removal in larvae of *Rana pipiens*. *J. exp. Zool.* **24**, 499–519.

Boschwitz, D. (1957). Thyroidless tadpoles of *Pelobates syriacus* O. Boettger. *Copeia*, **4**, 310–11.

—— (1960). The ultimobranchial body of the Anura of Israel. *Herpetologica*, **16**, 91–100.

Eggert, B. (1938). Der ultimobranchiale Körper d. Knochenfische. *Z. Zellforsch.* **27**, 754–63.

Getzowa, S. (1907). Über die Glandula parathyroidea, intrathyroidale Zellhaufen derselben und Reste des postbranchialen Körpers. *Virchows Arch.* **188**, 181–235.

—— (1911). Zur Kenntnis des postbranchialen Körpers. *Virchows Arch.* **205**, 208–57.

Gorbman, A. (1947). Functional and morphological properties in the thyroid gland, ultimo-branchial body, and persisting ductus pharyngiobranchialis IV of an adult mouse. *Anat. Rec.* **98**, 93–101.

Lynn, W. G., & Wachowsky, H. E. (1951). The thyroid gland and its functions in cold-blooded vertebrates. *Quart. Rev. Biol.* **26**, 123–68.

Mertens, R. (1947). *Die Lurche und Kriechtiere des Rhein-Main Gebietes*, p. 68. Frankfurt a. M.: Waldemar Kramer.

Saxén, L., & Toivonen, S. (1955). The development of the ultimobranchial body in *Xenopus laevis* Daudin and its relation to the thyroid gland and epithelial bodies. *J. Embryol. exp. Morph.* **3**, 376–84.

Schaefer, K. (1938). Morphologische und physiologische Untersuchungen am ultimobranchialen Körper von *Triturus vulgaris* und *Triturus alpestris*. *Z. wiss. Zool.* **151**, 22–38.

Steinitz, H., & Stone, L. S. (1954). Observations on the ultimobranchial body in the adult newt. *Triturus v. viridescens*. *Anat. Rec.* **120**, 435–48.

Sterba, G. (1950). *Über die morphologischen und histogenetischen Thymusprobleme bei* Xenopus laevis *Daudin, nebst einigen Bemerkungen über die Morphologie der Kaulquappen*, pp. 1–54. Berlin: Akad. Verlag.

Watzka, M. (1933). Vergleichende Untersuchungen über den ultimobranchialen Körper. *Z. mikr.-anat. Forsch.* **34**, 485–533.

EXPLANATION OF PLATE

FIG. 1. Ultimobranchial body of *Bufo* after metamorphosis. Its diameter, 115 μ; section 8 μ thick; 4 mm. obj.; $\times$290. Note typical follicles, parafollicular cells, and capillaries.

FIG. 2. Ultimobranchial body of *Bufo* 14 days after hypophysectomy. Its diameter, 110 μ; section 10 μ thick; 4 mm. obj.; $\times$290. Follicles are compressed and mostly indistinguishable from parafollicular cells. Wide capillaries. Compare with control (fig. 1).

FIG. 3. Ultimobranchial body of *Bufo* tadpole (total length 32 mm.) 31 days after complete thyroidectomy. Section 8 μ thick; 8 mm. obj.; $\times$250. Three distinct follicles surrounded by capsule with melanophores. Controls have only one follicle.

FIG. 4. Ultimobranchial body of postmetamorphic *Rana* 38 days after partial thyroidectomy. Diameter of ultimobranchial body, 210 μ; section 10 μ thick; 4 mm. obj.; $\times$290. Pseudostratified epithelium. Unusual amount of protruding and detached cells. Lumen greatly enlarged and capillaries wide: signs of hypertrophy.

FIG. 5. Ultimobranchial body of postmetamorphic *Rana* 111 days after hypophysectomy. Diameter 176 μ; section 8 μ thick; 4 mm. obj.; $\times$330. Epithelium single-layered. No coagulum in the narrow lumen. Capillaries reduced, capsule thin: signs of atrophy.

FIG. 6. Ultimobranchial body of postmetamorphic *Rana* 54 days after hypophysectomy and 15 days after thyroidectomy. Diameter 190 μ; section 10 μ thick; 4 mm. obj.; $\times$290. Effect of hypophysectomy: single-layered epithelium of large follicle with few protruding nuclei. No coagulum. Effect of thyroidectomy: lumen is secondarily expanded. The small follicle shows renewed activity: columnar epithelium, eosinophilic coagulum including cells.

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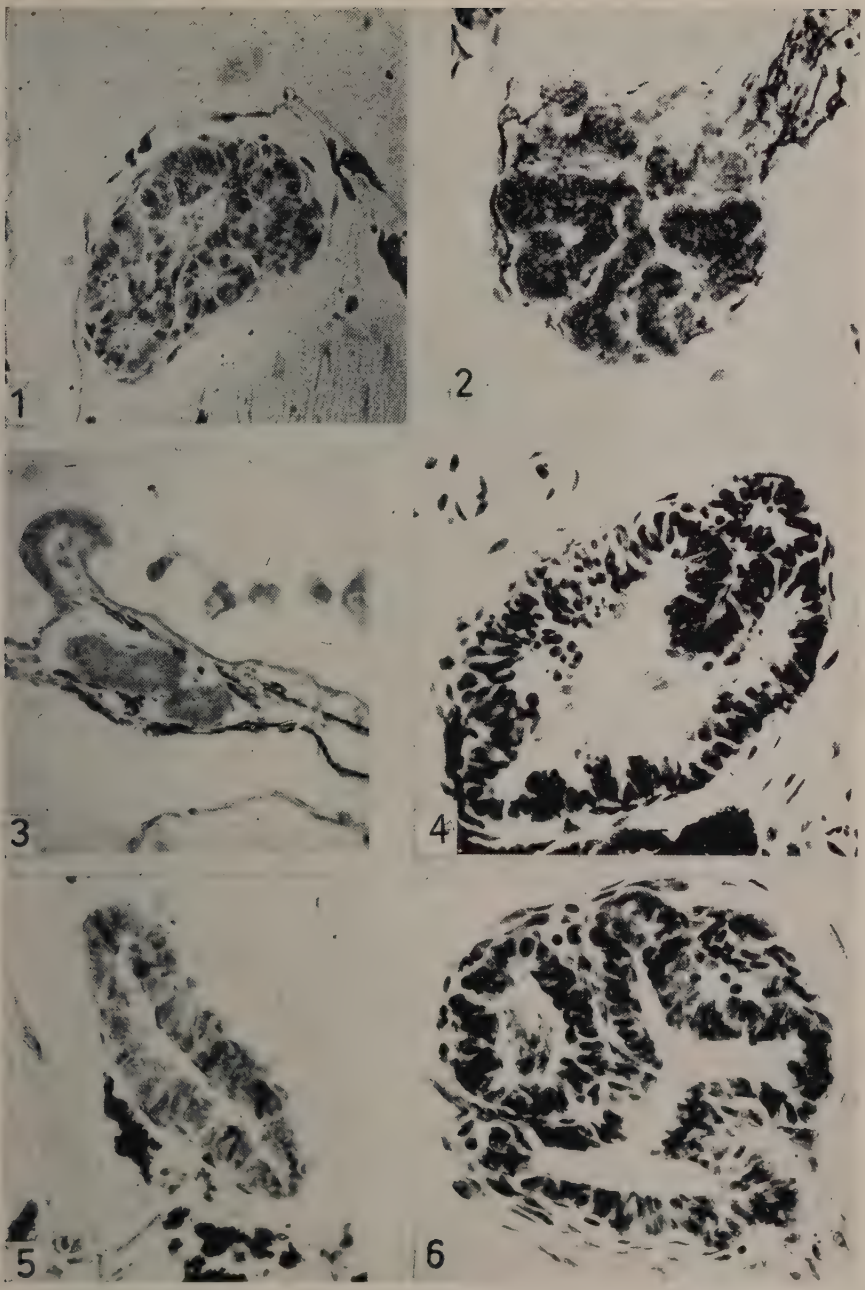

D. BOSCHWITZ

A Description of the Technique for Nuclear Transplantation in *Xenopus laevis*

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WITH PLATE

INTRODUCTION

A METHOD by which nuclei can be successfully transplanted into Amphibian eggs was first worked out by Briggs & King (1952) for the eggs of *Rana pipiens*. *Xenopus laevis* is an atypical Anuran since its eggs can be obtained throughout the year, and the resulting embryos can be reared to maturity within 12 months under laboratory conditions. Because of these advantages we have used *Xenopus* for nuclear transplantation experiments. Though the principle of Briggs & King's technique has been followed, differences between the eggs of *Rana* and *Xenopus* have made it necessary to modify their technique before it can be satisfactorily applied to the eggs of *Xenopus*. The purpose of this publication is first to give technical details of these modifications, and secondly to discuss the extent to which they might affect a direct comparison of the results of transplantations in *Rana* and *Xenopus*.

TECHNIQUE

Apparatus

A low-power binocular microscope giving a magnification of between $\times 25$ and $\times 50$ has been found suitable for transplanting nuclei. A syringe capable of delivering small quantities of fluid is required. We have used an 'Agla' micrometer syringe (Burroughs Wellcome & Co., London), in which a fairly strong spring was fitted to the shaft of the plunger, controlling the uptake of fluid. The needle of the syringe is connected to a micropipette by polythene tubing of about 1-mm. bore. The micropipettes are constructed as follows. Lengths of thin-walled glass tubing are cleaned chemically and thoroughly washed out. They are then drawn out to an internal diameter of 20 to 50 μ as required. The syringe, tubing, and micropipette are all filled with liquid paraffin (B.D.H., Ltd.); this has been found to give superior control to that given by air or water. It does not appear to have any harmful consequences on either the donor cell

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or recipient egg, with which it should not in any case come into contact. The pipette is held and controlled by a Singer 'Microdissector' (Plate, fig. A). This instrument reduces all hand movements by a factor of about 4, and does not invert movements in any direction. Direct movement is particularly valuable in the vertical plane.

The source of ultra-violet irradiation which we have used for inactivating the egg pronucleus is a 100-W medium-pressure mercury arc lamp made by Hanovia Ltd., Slough, England. This source gives an appreciable emission at all wavelengths from 2,100 Å to 3,200 Å, as well as visible light. The lamp is mounted horizontally, giving a downwardly directed beam on to the eggs, which are placed 13 cm. below the bulb. A quartz condenser, 4 cm. above the eggs, focuses the beam on to an area about 2 cm. in diameter.

The nuclear marker

A nuclear marker (Elsdale, Fischberg, & Smith, 1958; Fischberg & Wallace, 1960) has proved most helpful for our nuclear transplantation experiments. Various uses to which this marker has already been put have been described in recent publications from this laboratory (Fischberg, Gurdon, & Elsdale, 1958;

TABLE 1

The use of the Xenopus *nuclear marker for determining the origin of nuclei in transplant-embryos*

Donor nuclei from the 1-nucleolated strain are transplanted into recipient eggs from normal 2-nucleolated individuals

	Number of nucleoli		
Ploidy	1	2	3
Haploid	Parthenogenetic development of haploid egg pronucleus. Less than 1 per cent.		
Diploid	Development of transplanted nucleus only. 60–100 per cent.	Parthenogenetic development of diploid egg nucleus. Less than 1 per cent.	
Triploid		Haploid egg nucleus combined with diploid transplanted nucleus. Less than 1 per cent.	
Tetraploid		Doubled transplanted nucleus. Up to 40 per cent. (Gurdon, 1959).	Diploid egg nucleus combined with transplanted nucleus. Less than 1 per cent.

Gurdon, 1960; Blackler, 1960). Diploid nuclei of wild-type *Xenopus* each have two nucleolar organizers, and usually have two nucleoli; however, a variable proportion of such nuclei (average 30–50 per cent.) have only one nucleolus

resulting from fusion of the nucleolar material of the two organizers. The nuclear marker consists of a mutation causing inhibition or loss of the nucleolar organizer, of which there is one in every haploid set of chromosomes. Thus nuclei of diploid individuals heterozygous for this mutation have only one nucleolus, and may therefore be easily distinguished from nuclei of normal diploids, the majority of which have two nucleoli. In all other respects individuals heterozygous for this mutation are entirely normal. Animals homozygous for this mutation possess no proper nucleoli, but have small droplets of nucleolar substance which are of irregular size and number. However, homozygotes are inviable and die before feeding.

For nuclear transplantation experiments it has been customary to transplant marked donor nuclei into eggs of unmarked female frogs. Assuming that the ploidy and nucleolar number of a transplant-embryo is known, the origin of its nuclei is immediately apparent (see Table 1). The nuclear marker can be rapidly identified under the phase-contrast microscope.

The preparation of donor cells

The developmental stages of donor embryos have been identified according to the Normal table for *X. laevis* of Nieuwkoop & Faber (1956).

The dissociation of donor cells has been carried out by the methods of King & Briggs (1955). We usually use Barth's saline solution 'X' (Barth & Barth, 1959) instead of Niu and Twitty's solution, but the results are very little, if at all, affected by this change. Many tissues, especially those of younger embryos, can be dissected away from the rest of the embryo without the help of trypsin. The tissue is placed in Barth's solution 'X', in which calcium and magnesium are replaced by 5×10^{-4} M versene. The time required to bring about dissociation in versene increases as the age of the donor embryo increases. For early stages such as blastulae, 10 minutes of versene is sufficient. Longer periods of 20 minutes or more are needed for later stages. Dissociated cells can be kept without harm in normal strength Barth solution, phosphate-buffered to about pH 7·7, for over half an hour before being transplanted. When dissociated they are transferred to an agar-covered slide with at least 1 ml. of Barth solution on it. To prevent evaporation altering the strength of the saline solution, the slide carrying the donor cells should only be placed in the beam of the microscope lamp when drawing the cell into the pipette.

The preparation of recipient eggs

Adult females of *Xenopus* can be induced to ovulate several times a year by injection of gonadotropic hormone. We have used the commercial product 'Gonan' (B.D.H., Ltd.) or 'Pregnyl' (Organon Laboratories, Ltd.), and find that a dose of about 300 units is suitable for females and one of 150 units for males (the latter should have black nuptial pads on the forearm before injection).

The dose is varied according to the size of the animals and their condition. The presence of a male is not necessary to induce a hormone-injected female to lay eggs. It has been shown (Gurdon, 1960*b*) that eggs used as recipients for nuclear transplantation give results which vary not only from one frog to another, but also from one ovulation to the next of the same frog. No way has yet been found of improving the quality of eggs, and we have no evidence that the eggs of frogs imported from Africa give consistently better results than the eggs of those reared in the laboratory.

Before transplanting a donor nucleus into an egg, it is necessary to remove or inactivate the egg pronucleus. In frogs of the genus *Rana* and in newts this can be reliably done by lifting the egg nucleus and a little surrounding cytoplasm out of the egg with a needle (Porter's technique, 1939). This method is used by Briggs & King for their nuclear transplantation experiments with *Rana*. The great strength and elasticity of the vitelline membrane of *Xenopus* eggs prevents this method being used both reliably and harmlessly for the eggs of this species (Gurdon, 1960*a*). We have found that a short exposure (less than 1 minute) of the animal pole of an egg to ultra-violet irradiation inactivates the egg pronucleus but does not damage the egg in any other respect. Another very considerable advantage of this ultra-violet treatment is that it renders the vitelline membrane penetrable to a micropipette without destroying it, and at the same time destroys the jelly over the animal pole of the egg. It may also sterilize the injection area. Unless weakened in this way, the vitelline membrane cannot be penetrated by a pipette without damaging the egg. The effects of ultra-violet irradiation on *Xenopus* eggs form the subject of another publication (Gurdon, 1960*a*), which describes the fate of the irradiated egg nucleus and considers the evidence that the ultra-violet does not damage the egg cytoplasm. The main reason for this latter belief is that a considerable increase in u.v. dose does not increase the abnormalities of transplant-embryo development.

Best results are obtained if eggs are not used for transplantation immediately after laying, but are left in water for about 15 minutes (Gurdon, 1960*b*). Eggs to be irradiated are placed dry, about six at a time, on a glass slide. Free water must be removed so that the egg remains with its animal pole facing upwards. The slide carrying the eggs is then placed under the ultra-violet beam for the appropriate amount of time (between 20 and 50 seconds according to the particular ovulation). The eggs are now ready for the injection of the donor nucleus.

The transplantation procedure

The disaggregated donor cells on an agar-covered slide can be seen very easily if all light coming from below the slide is cut out and strong illumination from above is used (Plate, fig. B). There is some variation in the size of cells from various regions of a donor embryo, and a cell is chosen which is just too big to fit into the end of the pipette, so that the cell-wall is broken when the cell is sucked up (Plate, fig. B). The cell cytoplasm should still completely surround

the nucleus, which must not be greatly distorted by this treatment. This part of the technique follows the technique of Briggs & King (1952).

The slide carrying the irradiated eggs (still not surrounded by fluid) and that carrying the donor cells can conveniently be placed on a larger glass slide. This enables the donor cells or recipient eggs to be quickly placed in the field of view as required. Before the donor cell is drawn into the pipette, a small air bubble should be sucked into it so as to separate the paraffin from the saline solution (Plate, fig. B). This allows the passage of fluid out of the pipette to be accurately observed when the donor cell has passed out of sight (Plate, fig. C). The position of the air bubble must, of course, be adjusted each time a new donor cell is sucked up. The pipette is inserted into the egg so that its tip which contains the donor cell comes to lie somewhere between the centre of the egg and its animal pole. While watching the air bubble the syringe control is turned so that the donor cell and a small amount of saline solution (but not the air bubble) is deposited near the centre of the egg. If this is done carefully and if the u.v. dose has been judged correctly, the egg will heal up very quickly after withdrawal of the pipette (Plate, figs. D, E). It is desirable to inject a small amount of saline solution with the donor cell because it helps to ensure that the cell is not damaged by the end of the pipette, whilst at the same time it does not affect development. During the operation the pipette has been controlled with the help of the microdissector. The average time required for the injection of one egg with a nucleus is about 1 minute. Thus, if six eggs on one slide are irradiated and injected, the eggs will remain uncovered with fluid for about 10 minutes. If the operation takes longer, fewer eggs should be done at one time. If eggs are left uncovered by water for too long, the jelly and vitelline membrane may dry on to the egg surface, causing abnormal development or preventing development altogether.

The culturing and scoring of transplant-embryos

As soon as the eggs on one slide have all been injected with a nucleus, the slide is placed in a small Petri dish containing full-strength Niu & Twitty's solution. The eggs are left in this solution for a few hours because this facilitates the healing of the injection wound. This solution is replaced by one-tenth strength Niu & Twitty's solution some time during cleavage. Shortly before hatching the embryos are placed in tap-water from which excess chlorine has been allowed to evaporate. When tadpoles are ready to feed they are placed in 50-ml. tubes and fed on a weak suspension of powdered nettles (*Urtica* sp.). They are changed to a larger bottle (about 3 tadpoles per litre of water) as they grow, but are fed on nettle-powder until metamorphosis, which occurs about 2 months after laying. We experience very little mortality between the beginning of feeding and metamorphosis, and have not found it necessary to use antibiotics at any stage. After metamorphosis frogs are fed on *Tubifex*, and later on liver.

Many transplant-embryos develop abnormally, and the following procedure

is followed when it is clear that an embryo will not differentiate any further. It is drawn under a camera lucida; then a small part of it is lightly squashed on a slide under a coverslip. The number of nucleoli per nucleus can be quickly counted under the phase-contrast microscope (Elsdale, Fischberg, & Smith, 1958). Chromosome numbers can be determined after leaving the tissue in aceto-orcein for about 1 minute before squashing. The rest of the embryo may then be fixed for sectioning. If an embryo is not required for sectioning, the whole of it may be squashed so that the ploidy and nucleolar number can be estimated in different regions. If the embryo is old enough to have a tail, part of this may be removed and stained in haemalum, which shows the nucleoli and chromosomes clearly.

THE MAIN DIFFERENCES IN THE NUCLEAR TRANSPLANTATION TECHNIQUES FOR *XENOPUS* AND *RANA*

The most obvious difference between Briggs & King's technique for *Rana* and our modifications of it for *Xenopus* concerns the method of recipient egg enucleation. With *Rana* the egg nucleus is removed from the egg, while in *Xenopus* it is irradiated with ultra-violet and then allowed to degenerate in the egg cytoplasm. The fate of the irradiated egg nucleus in *Xenopus* has been followed, and there is no reason to believe that this or the other consequences of the ultra-violet treatment affect the development of the transplanted nucleus in any way (Gurdon, 1960*a*); this question is discussed further by Briggs & King (1960) and Gurdon (1960*c*).

Another modification which we have introduced concerns the *Xenopus* nuclear marker. The presence of this marker is not associated with any abnormalities, but gives proof that the nuclei of transplant-embryos are derived from the injected nucleus alone.

Other differences also exist which are of considerable practical importance but which do not affect the results. For instance, *Xenopus* eggs are left uncovered by saline solution for about 10 minutes during transplantation; this only causes damage if the eggs are allowed to become dry on the surface; but eggs in which this has happened can easily be recognized and thrown away.

When comparing nuclear transplantation experiments in *Rana* and *Xenopus*, variation in the quality of recipient eggs must be considered. We have found that nuclei from the same donor embryo, when transplanted into the eggs of different females, can give very different results. It seems that some of our abnormal transplant-embryos may be attributed to poor quality of recipient eggs; this might be due to the fact that it is not possible to give *Xenopus* the same conditions in the laboratory as they enjoy in the wild.

One may conclude that, with the possible but unlikely exception of egg nucleus degeneration in *Xenopus*, none of the differences in technique between *Rana* and *Xenopus* influence the results of experiments with these species.

Differences in the results should therefore represent real differences between the species and not differences in technique. This conclusion applies only in principle, and does not take account of variation due to any inconsistency with which the technique is carried out on different occasions or by different workers. The effect of varying certain parts of the technique, more than would happen under normal circumstances, is described elsewhere (Gurdon, 1960*b*).

SUMMARY

1. Technical details are given of a method of transplanting embryonic nuclei in *X. laevis*.

2. This technique is based on Briggs & King's method for *R. pipiens*. Owing to certain differences between the eggs of *Rana* and *Xenopus*, their technique has been modified for *Xenopus*.

3. The main modification for *Xenopus* concerns the use of ultra-violet irradiation instead of enucleation by a needle.

4. Differences between transplantation techniques for *Rana* and *Xenopus* are unlikely to account for the differences in experimental results obtained with these species.

RÉSUMÉ

Description de la technique de transplantation du noyau chez Xenopus laevis

Des détails techniques sont donnés sur une méthode de transplantation des noyaux embryonnaires chez *Xenopus laevis*. Cette technique est basée sur la méthode employée par Briggs & King chez *Rana pipiens*. A cause de certaines différences entre les œufs de *Rana* et de *Xenopus*, la technique a été modifiée pour *Xenopus*. La principale modification concerne l'utilisation des rayons ultra-violets à la place de l'énucléation par une aiguille. Les différences entre les techniques de transplantation utilisées chez *Rana* et *Xenopus* n'expliquent vraisemblablement pas dans les différences entre les résultats obtenus chez ces deux espèces.

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REFERENCES

Barth, L. G., & Barth, L. J. (1959). Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. exp. Morph.* **7**, 210–22.

Blackler, A. W. (1960). Transfer of germ-cells in *Xenopus laevis*. *Nature*, **185**, 859–60.

Briggs, R., & King, T. J. (1952). Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc. nat. Acad. Sci. Wash.* **38**, 455–63.

Briggs, R., & King, T. J. (1960). Nuclear transplantation studies on the early gastrula (*Rana pipiens*). I. Nuclei of presumptive endoderm. *Devel. Biol.* **2,** 252–70.

Elsdale, T. R., Fischberg, M., & Smith, S. (1958). A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp. Cell. Res.* **14,** 642–3.

Fischberg, M., Gurdon, J. B., & Elsdale, T. R. (1958). Nuclear transfer in Amphibia and the problem of the potentialities of the nuclei of differentiating tissues. *Exp. Cell Res.* (Suppl.), **6,** 161–78.

—— & Wallace, H. (1960). The cell nucleus. In symposium, ed. Holmes. London: Butterworth, 30–34.

Gurdon, J. B. (1959). Tetraploid frogs. *J. exp. Zool.* **141,** 519–44.

—— (1960*a*). The effects of ultraviolet irradiation on uncleaved eggs of *Xenopus laevis*. *Quart. J. micr. Sci.* **101,** 299–312.

—— (1960*b*). Factors affecting the abnormal development of transplant-embryos in *Xenopus laevis*. *J. Embryol. exp. Morph.* **8,** 327–40.

—— (1960*c*). The developmental capacity of nuclei taken from differentiating endoderm cells of *Xenopus laevis*. *J. Embryol. exp. Morph.* **8,** 505–26.

King, T. J., & Briggs, R. (1955). Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. *Proc. nat. Acad. Sci. Wash.* **41,** 321–5.

Nieuwkoop, P. D., & Faber, J. (1956). *Normal Table of* Xenopus laevis (*Daudin*). Amsterdam: North Holland Publishing Company.

Porter, K. R. (1939). Androgenetic development of the egg of *Rana pipiens*. *Biol. Bull. Wood's Hole*, **77,** 233–57.

EXPLANATION OF PLATE

Fig. A. Photograph of the transplantation apparatus, showing a 'Singer' microdissector, a microscope, and an 'Agla' syringe with polythene tubing. Illumination is provided by a small spot-lamp (not shown).

Fig. B. Disaggregated cells of a *X. laevis* late blastula, one of which has just been sucked into the end of the micropipette; the air-bubble marker can be seen in the shaft of the pipette. ×25.

Fig. C. The micropipette is seen inserted into the recipient egg. The donor cell has been injected and the air-bubble marker (dark) is seen where the shaft of the pipette penetrates the egg. ×25.

Fig. D. A 2-cell stage of a successfully transplanted egg. The penetration wound has completely healed and can no longer be seen. ×25.

Fig. E. Another transplant-embryo at the morula stage. The small exovate from the injection (lower right of picture) has separated from the surface of the 'egg', and will not interfere with further development. ×25.

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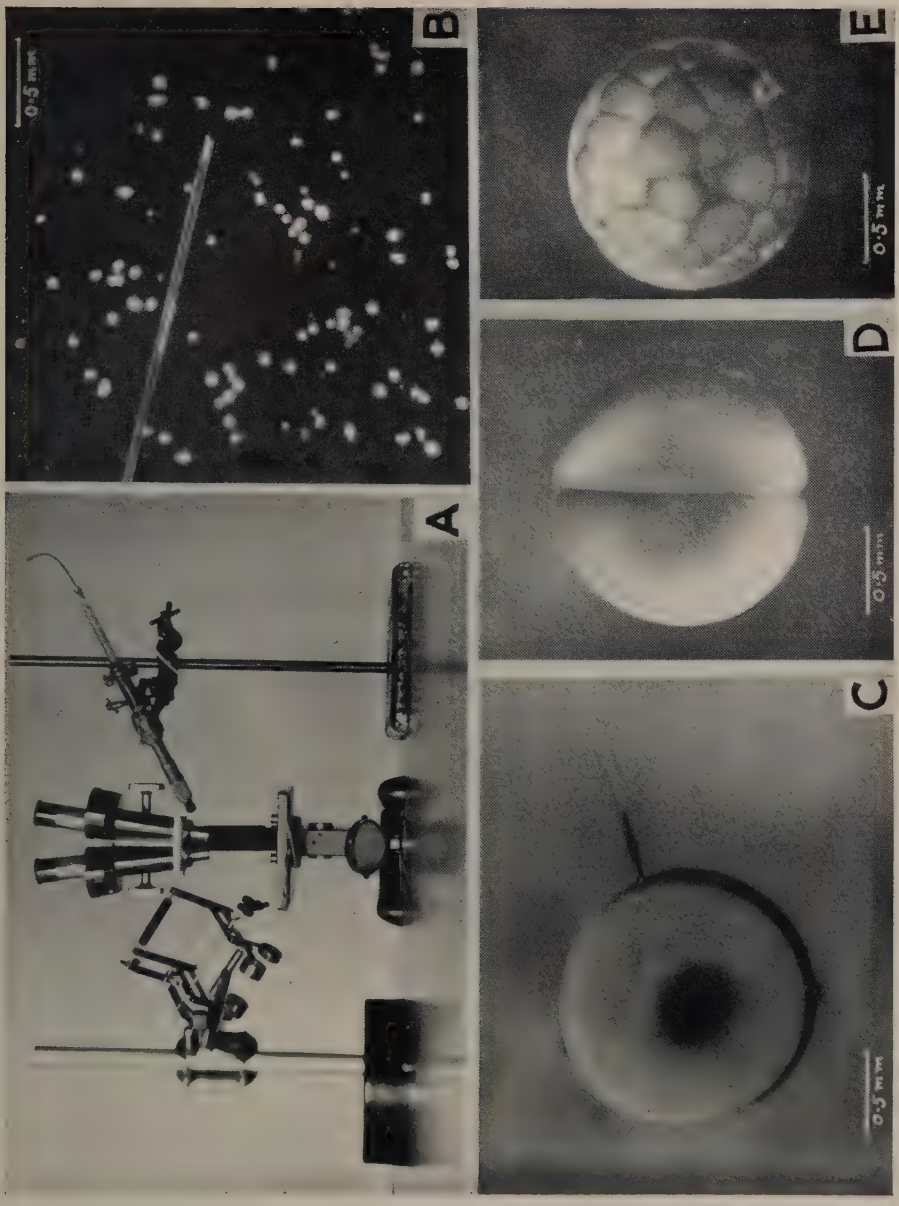

T. R. ELSDALE, J. B. GURDON, *and* M. FISCHBERG

Embryonic Responses to Structurally Related Inhibitors

II. The Effect of Substituents

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WITH TWO PLATES

INTRODUCTION

SUBSTITUTED benzotriazoles (Bt) have been shown to bring about interesting inhibitory effects on *Rana pipiens* embryos of 2-cell to tail-bud stages (Liedke, Engelman, & Graff, 1954, 1955, 1957*a*, 1957*b*). These benzotriazoles did not have selective cytotoxic effects on sensitive embryonic structures as similarly substituted benzimidazoles (Bz) and quinoxalines (Q) invariably did. The latter compounds, Bz and Q, were most active against younger stages, especially those in cleavage. On the other hand, it was found that the susceptibility to the benzotriazoles increased with age of embryo; more differentiated stages were affected most. The type of response was determined by the parent structure, but certain substituents, the nitro group in particular, appeared to enhance the magnitude of the effect. The activating effect of the nitro group was in turn modified to varying degree by an accompanying methoxy, hydroxy, or amino group.

It appeared desirable to study these modifying effects further by employing derivatives of the three classes of compounds, in which (*a*) a chlorine atom replaced the nitro group, and (*b*) in which the nitro group is accompanied by either a chlorine atom, a methyl, a carboxyl, or a second nitro group. Embryos at the 2-cell, blastula, neurula, or tail-bud stages and tadpoles (stages 3, 8, 14, 18, and 25, according to Shumway, 1940) were exposed to the test compounds. In addition to the Bz, Bt, and Q compounds, four other compounds (2,3-diamino-5-nitro-benzoic acid, 3,4-diamino-5-nitro-benzoic acid, 3,4-diamino-2-nitro-anisole, and 5-chloro-3-nitro-*o*-phenylene diamine) were tested similarly.

MATERIAL AND METHODS

The methods were the same as those described in an earlier communication (Liedke, Engelman, & Graff, 1955). For a given experimental series, embryos from the same egg-batch were used. Exposure to the compounds (10 embryos

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per 20 ml. of test solution per stender dish) was started when the embryos had developed to stages 3, 8, 14, and 18. All solutions were made up in 10 per cent. Ringer solution and maintained at approximately pH 6·7 (phosphate buffer). At the concentration used (50 μg./ml.) the compounds have little or no effect on pH. Temperature varied from 20 to 22° C. in the experiments using continuous exposure, and from 22 to 24° C. in the experiments where the length of exposure was varied. Instances of low solubility of test compounds are noted in the table.

Egg-batches from 10 different females were used in the experimental series. Different egg-batches varied somewhat in their response to the compounds, but the results were consistent when all the compounds were tested on the same batch. Varying lengths of exposure were employed with 6 batches of eggs also, in conformity with our previously reported finding that the severity of effect depends on the length of exposure as well as on concentration.

RESULTS

In Table 1 are listed a total of 34 compounds employed in the experiments at a concentration of 50 μg./ml. (except where solubility was limited) in the experiments on young embryos, and at a considerably lower concentration in the experiments on tadpoles (see below). Six of these compounds had been used in earlier experiments (Liedke, Engelman, & Graff, 1954, 1957*b*) and were used again to broaden the base of comparison.

Experiments on embryos in 2-cell, blastula, neurula, and tail-bud stages

Continuous exposure

The data in Table 1 show that:

(1) Toxicity was markedly enhanced where either the benzotriazole or benzimidazole nucleus was nitro-substituted, as had been found before. Unsubstituted quinoxaline is highly toxic in itself, and nitro substitution affected it but little.

(2) Chlorine substitution enhanced toxicity of the parent compound much less than did nitro substitution, except at S. 3 with 5-Cl-Bz, which was highly toxic.

(3) Double substitution with nitro and chloric groups or with two nitro groups produced variable toxicity depending on the parent compound.

(4) The introduction of a methyl group to a nitro-substituted compound usually decreased the toxicity to older stages, but not at all when stage 2 embryos were treated.

(5) The addition of a carboxyl group to a number of compounds was most decisive, completely abolishing toxicity in most instances.

Not listed in Table 1 are four compounds which were also tested. Three of these had no effect on the embryos, namely: 2,3-diamino-5-nitro-benzoic acid, 3,4-diamino-5-nitro-benzoic acid, and 3,4-diamino-nitro-anisole; but continuous

exposure to 50 μg./ml. of 5-chloro-3-nitro-*o*-phenylene diamine arrested 2-cell stages at S. 12–15, blastulae at S. 15, neurulae at S. 18–19, and tail-buds at S. 20.

TABLE 1

Stage of arrest in development of Rana pipiens *embryos after continuous exposure to 50 μg./ml. of various substituted benzimidazoles* (Bz) *and benzotriazoles* (Bt)

References†	*Substituents on parent compound*	*Embryonic stage (S.) at which development stopped when exposed*							
		2-cell, S. 3		*Blastula, S. 8*		*Neurula, S. 14*		*Tail-bud, S. 18*	
		Bz	Bt	Bz	Bt	Bz	Bt	Bz	Bt
	None*	15	n	21	n	24	n	24	n
1, 2	5-N*	8	6–7	12–15	11–12	15–16	14	19	n
8	4-Cx-6-N	n	n	n	n	n	n	n	n
3, 4	4,6-diN	7	3–4	12	11	15	14	18	18
5, 6	5-Cl	4	8–9	15	12	19–20	16	20	18
7, 8	6-Cl-4-N	7–8	5–4	14	10–11	16	14	19	18
8	6-Me-4-N**	4–6	6–7	21–23	11–12	23–24	14–15	24	18
8	2-Me-6-N-4-Cx	n		n		n		n	
11	2-Me-11-N-6-Cx	n		n		n		n	
9	5-OH-4-N*	19	11–12	23	12–14	23	16	23	
9	2-Me-6-MO-4-N	11		23–24		23–24		23–24	
9	2-Me-5-MO-4-N	19		n		n		n	

		Substituents on quinoxalines (Q)			
		2-cell, S. 3	*Blastula, S. 8*	*Neurula, S. 14*	*Tail-bud, S. 18*
	None	5–6	16–17	20	22
5	6-N*	4–5	15–16	19–20	21–23
8	7-Cl-5-N	3–4	11	15	19
8	7-Me-5-N	4–5	15–16	19	21
10	2,3-diMe-6-N	15–17	16–17	19–20	19–20
9	2,3-diMe-6-MO-5-N	14	14	18	19
8	2,3-diMe-7-Cl-5-N	15–17	16–17	18–19	19–20
8	2,3-diPh-7-N-5-Cx**	10–11	11	14	18
11	2,3-diPh-5-N-7-Cx**	11–12	12	15	18
11	2,3-diMe-5-N-7-Cx	n	n	n	n
8	2,3-diMe-7-N-5-Cx	n	n	n	n
9	6-MO-5-N	n	n	n	n
9	5-A-2,3-diMe-6-MO	n	n	n	n
9	5-AcA-2,3-diPh-8-MO	n	n	n	n

Abbreviations: n = normal development; AcA = Acetamido; A = amino; Me = methyl; Cx = carboxy; Cl = chloro; Ph = phenyl; MO = methoxy; N = nitro.

* Compound previously reported.

** Compounds of limited solubility.

† *References*:

1. Bamberger (1893).
2. Hofmann (1860).
3. Bahner *et al.* (1952).
4. Nietzki & Hagenbach (1897).
5. Fischer (1904).
6. Ullmann & Mauthner (1903).
7. Hoover & Day (1955).
8. Gillespie *et al.* (1960).
9. Gillespie *et al.* (1957).
10. Bost & Towell (1948).
11. Gillespie (unpublished work).

Varied length of exposure

Varied length of exposure (Table 2) using the same concentration (50 μg./ml.) at 22–24° C. generally confirmed the results given in Table 1, but it also permitted some finer distinction.

Table 2 shows that Cl-N-Bt is the most toxic compound, except at the 2-cell stage (S. 3) where 1 hour of exposure permits development to stage 11, while N- or Cl-N-Q-treated 2-cell stages are stopped already at S. 3–5. But longer exposure to Cl-N-Bt (2·8 hours) also stops development earlier, at S. 5–6.

TABLE 2

Stage of arrest in development of Rana pipiens *embryos after 1, 2, 8, or 24 hours of exposure to 50 μg./ml. of various substituted benzotriazoles* (Bt), *benzimidazoles* (Bz), *and quinoxalines* (Q)

Developmental stage at start of exposure	*Substitution*	*Embryonic stage at which development stopped when exposed for*											
		1 hour			*2 hours*			*8 hours*			*24 hours*		
		Bt	Bz	Q	Bt	Bz	Q	Bt	Bz	Q	Bt	Bz	Q
2-cell (stage 3)	none	n	n	n	n	n	8–10	n	n	4–5	n	15–18	4–5
	NO_2	n	n	3–5	n	n	3–5	12	11	3–4	8–9	8	3–5
	Cl-NO_2	11	n	3–4	6	n	3–4	5–6	19–21	3–4	5–6	12	3–4
	Cl	n	n	..	n	9	..	n	5–6	..	15–18	5–6	..
	diNO_2	n	n	..	n	n	..	15–17	10	..	7	8	..
Blastula (stage 8)	none	n	n	n	n	n	n	n	n	16–17	n	n	16–17
	NO_2	n	n	n	n	n	23	23	23	14–17	9–10	17–18	12–16
	Cl-NO_2	8	n	22	8	n	19–21	8	23-n	12–13	8	21–23	12
	Cl	n	n	..	n	22–23	..	24-n	16–18	..	23	16	..
	diNO_2	n	n	..	n	23–24	..	11	21–23	..	10	12	..
Tail-bud (stage 18)	none	n	n	n	n	n	n	n	n	n	n	n	23-n
	NO_2	n	n	n	n	n	n	21-n	n	n	18	19–21	22–23
	Cl-NO_2	18	n	n	18	n	21–23	18	19–24	19	18	19	18–19
	Cl	n	n	..	n	n	..	n	n	..	23-n	22–24	..
	diNO_2	n	n	..	20-n	n	..	18	n	..	18	18	..

Table 2 also shows that N- or Cl-N-substituted quinoxaline is more toxic than Q (i.e. it requires only 1 hour of exposure, while Q requires 2–8 hours to arrest at stages 4–5). On exposure at later stages (S. 8, 18) this difference is even more apparent, and it is also seen that Cl-N-Q is more toxic than N-Q. It is seen, furthermore, that at later stages, S. 8, and even more at S. 18, longer exposures are required for Q-compounds than at S. 3, while almost the reverse is true for substituted Bt-compounds. However, it should be noted that Bt-compounds (except the double substituted diN- or Cl-N ones) require longer exposures than Q- or most Bz-compounds at all stages, i.e. at least 8 hours of treatment are required. Nevertheless, allowing 8–24 hours of treatment, even the single substituted benzotriazoles acted more severely at later than at earlier developmental stages.

The diN-substituent is generally more toxic than single N- in Bt and Bz (cf. columns 8 and 24 hours in Table 2). Cl- in place of N- is definitely less toxic in Bt, but that is reversed in Bz, and the order of toxicity is Cl-Bz > N-Bz, particularly at stages 3 and 8. This difference is more apparent in Table 2 than in Table 1. Nevertheless, paradoxically, the added Cl- or double substitution (Cl-N-Bz) is less toxic than either of the single ones (N-Bz or Cl-Bz). On the other hand, double Cl-N in Bt is extremely toxic, and Cl-N-Bt is the most toxic compound of all the ones used, as will be shown below in experiments with 11-mm. tadpoles (Table 3). One could have expected the opposite, namely, that Cl-N-Bz rather than Cl-N-Bt would be the most toxic compound if it were a matter of simply adding 'more toxic Cl-' to N-Bz, and 'less toxic Cl-' to N-Bt.

Experiments with 10–11-mm. tadpoles (S. 25)

For each experimental series tadpoles from the same egg-batch were exposed to the three unsubstituted parent compounds and the N-substituted ones, and also to Cl-N-Bt (5 tadpoles per 20 ml. of test solution per large stender dish, or 10 per 40 ml. per finger-bowl).

TABLE 3

The effect of Cl-NO_2 *or* NO_2-*substituents in benzotriazole* (Bt), *benzimidazole* (Bz), *or quinoxaline* (Q) *on* Rana pipiens *embryos* (*stage 25*)

Compound	*Concentration μg./ml.*	*Length of exposure*	*Abnormal, died after*		*Normal, survival*
			4–20 hours	*2–5 days*	
Cl-N-Bt	1	3 hours			+
	2	2 hours	+		
	5	15 min.	+		
	20	10 min.	+		
N-Bt	20	30–50 min.			+
	20	70 min.	+		+
	20	90 min.	+		
	5	continuous	+	+	
N-Bz or N-Q	20	5–6 hours		+	+*
	20	continuous		+	
Bt	500	3 hours			+
	500	continuous	+		
Bt, Bz, or Q	200	continuous		+	

* Some abnormal survival with slight oedema and loss of eye pigmentation, especially in N-Q-treated tadpoles.

The great toxicity of the substituted benzotriazoles on older embryos became rather troublesome in the work with these tadpoles (S. 25) which are 6 days older than the tail-bud embryos (S. 18) used in the above-described experiments (Tables 1, 2). Survival was irregular and unpredictable even at very low concentrations of N-Cl-Bt or N-Bt (Table 3). The tadpoles were normal and active after the brief exposure to N-Cl-Bt or N-Bt, when they were rinsed of the test solution and transferred to 10 per cent. Ringer solution. Sometimes it seemed that their motility was even greater or slightly spastic compared to the controls.

Nevertheless, motility often ceased within 2–6 hours after treatment, and very soon thereafter the heart stopped beating. On very rare occasions some individuals survived and continued normal growth like the controls. Neither the volume of blood in the heart nor gill circulation can be observed in these tadpoles because of overgrowth of the operculum. However, in simultaneous identical experiments with younger tadpoles (S. 23) it was noticed that the gill circulation had become more sluggish and sometimes stopped during the first day after treatment.

Such sudden death, or only occasional survival of some individuals, was not found with N-Bz or N-Q. Rather the same concentration (20 μg./ml.) and even continuous exposure to N-Bz or N-Q permitted survival from 2 to 5 days, and for longer if exposure was for 5–6 hours (compared to 1–2 hours with N-Bt) as seen in Table 3. Only slight or no oedema developed in these tadpoles exposed to 20 μg./ml. N-Bz or N-Q for 5–6 hours. Three days later the eyes showed some loss of pigment, particularly in the N-Q-treated tadpoles, which were not studied histologically. It is therefore not possible to state how much necrosis and possible regeneration occurred. Loss of eye pigment can indicate regeneration if necrosis is stopped, and the tadpole is not oedematous. With continuous exposure to 20 μg./ml. N-Bz or N-Q the tadpoles became progressively oedematous and showed poor motility, and death occurred between 2 and 5 days after start of exposure. The same results were obtained with the three unsubstituted parent compounds when the concentration was increased tenfold, i.e. 200 μg./ml. of N-Bz, N-Bt, or N-Q. Tadpoles from these experiments (continuous exposure to 200 μg./ml. Bz, Bt, or Q, or to 20 μg./ml. of the N-substituted compounds) were studied histologically.

Histological observations

For histological study some tadpoles were killed 4 hours or 1, 2, or 3 days after initiation of the treatment. Harris haematoxylin, phosphatungstic acid haematoxylin, and eosin-azure stains were used after fixation in Bouin, Zenker, or Helly fluid. Embryos from some of the younger series were also prepared for histological study.

In previous work (Liedke, Engelman, & Graff, 1955, 1957*b*) the characteristic response of these younger embryos (stages 3, 8, 14, and 18) to Bt-compounds always involved a generalized effect, namely, sudden death, or delay in development leading to certain malformations depending on the developmental stage treated. A localized cytotoxic effect, like selective necrosis or enlargement of cells in sensitive embryonic structures, was not found in spite of abnormal development and some microcephaly. Such a localized cytotoxic response, on the other hand, always occurred with quinoxalines and benzimidazoles. In this study, however, a localized cytotoxic response was seen also in Cl-substituted benzotriazoles in these younger embryos (stages 3, 8, 14, and 18).

Histological study of 11-mm. tadpoles treated with all three compounds

always showed localized selective cellular necrosis in the eye, central nervous system, head cartilage, blood-cells, and the liver. The N-substituted compounds were more effective than the unsubstituted ones; N-substituted benzotriazole was more toxic than N-substituted benzimidazole or quinoxaline, and N-Cl-Bt was extremely toxic (Table 3).

One would expect localized cytotoxic responses in 11-mm. tadpoles to any interfering agent. The principal organ systems are present, although growth and differentiation still continue, and some structures, limbs, spleen, gonads, &c., have not as yet developed. The circulatory system functions and is now of primary importance, even though intracellular utilization of yolk still provides metabolites. The number of blood-cells increases constantly. It is, therefore, not surprising that not only the sensitive eye and nervous system, as in younger developmental stages, should be strongly affected, but also the blood-cells and liver.

Two or three days after exposure the muscular activity of the tadpoles was poorer and oedema had developed. The heart continued beating at a normal rate but the volume of blood seemed to be less. Unfortunately, gill-circulation changes could not be observed because of overgrowth of the operculum; however, shrinkage of gills was apparent in sections of some tadpoles.

The most severe effect prior to death was complete failure of the circulatory system and advanced necrosis in the liver. This is illustrated in Plate 1, fig. A, Plate 2, fig. F, which show transverse sections of a tadpole 3 days after continuous exposure to 0·020 μg./ml. of unsubstituted benzotriazole. When the circulation is disrupted, the lens becomes pressed into the eye-cup, while vascular spaces between the eye and brain, and capillaries in the brain, are obliterated, as a comparison between figs. A, B, C of Plate 1 shows. Not only is the number of blood-cells reduced, but necrotic blood-cells are everywhere evident, particularly in the liver. The liver-cells are also dying and the pronephric tubules have become distended (Plate 2, figs. E, F). At the same time, pycnotic nuclei are present in the cartilage of the head and the nerve-cord, but the most extensive necrosis and cell debris are found in the ventral portion of the brain. Most cells, particularly those in the eye, stain poorly (Plate 1, fig. A). All compounds elicited this severe cytotoxic response, but the effectiveness or toxicity varied greatly (N-Bt $>$ NQ or N-Bz $>$ Bt or Q), as seen in Table 3.

It was possible to trace the histological events which led to this severe cytotoxic response by fixing the tadpoles at different time intervals (4–72 hours after initiation of treatment with N-Bz, N-Bt, N-Q, Bt, or Q). The earliest abnormalities were noticed in the eye, usually on the first day after treatment, as seen in Plate 1, fig. C, which is a transverse section through the eye of a tadpole 1 day after exposure to 20 μg./ml. N-Q. A few pycnotic nuclei (*n*) are seen here, lying next to normal mitoses (*m*) in the iris angle, the zone of most rapid proliferation and earliest cellular differentiation. While normal tadpoles also have a few degenerating nuclei in the eye and central nervous system, such pycnotic nuclei

occurred more frequently in N-Q eyes, and earlier than blood-cell destruction. In this particular specimen, however, localized necrosis of a few blood-cells (*nB*) was seen in a few sections through the choroid plexus (Plate 2, fig. H). The pigment cells (P) are also affected and contracted. This effect on pigment cells occurred frequently with N-Q, even when there was no localized disturbance in the blood-cell population, but not with N-Bz nor Bt.

A more severe effect is usually found on the second or third day, as seen in Plate 1, fig. B, a transverse section through the eye region of a tadpole 2 days after exposure to 20 μg./ml. N-Bz. The number of necrotic cells (*n*) in the iris angle has now increased, mitotic figures are absent, and pycnotic nuclei are also scattered in the brain, adjacent to the ependymal layer. There is no visible involvement of blood-cells, in fact, the capillaries of brain and eye contain normal blood-cells, as Plate 1, fig. B shows. However, a few groups of degenerating blood-cells (*nB*) are scattered among the normal ones in the liver sinuses of this tadpole (Plate 2, fig. G).

Necrotic areas in the brain increased with time in size and number, extending to posterior levels, and pycnotic nuclei appeared in the head cartilage; the number of necrotic blood-cells increased in the liver sinuses and began also to appear in the pronephric sinuses. All gradations from slight to severe destruction of sensitive cells in the eye, the central nervous system, and cartilage, as well as of blood-cells, were found in tadpoles treated continuously with N-Q, N-Bz, or Bt, and this led finally to liver necrosis (Plate 1, fig. A; Plate 2, fig. F) as described above.

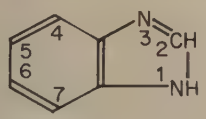
With shorter exposure (5–6 hours in 20 μg./ml. N-Q or N-BZ) only slight or no oedema developed; survival time was increased (Table 3). However, the results were always unpredictable with N-Bt, as described above. Histological study showed that some of the surviving N-Bt-treated tadpoles were normal; in other survivors of normal appearance necrotic cells in the eye or brain were still present 24 hours later (Plate 1, fig. D). This tadpole, however, has a normal liver (Plate 2, fig. E) with many mitotic cells.

DISCUSSION

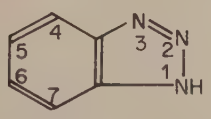
These experiments again illustrate the influence of type, number, and position of substituents on biological activity. The benzotriazoles, benzimidazoles, and quinoxalines shown in Text-fig. 1 are rather stable resonating structures. Their biological reactivity probably involves nitrogen atoms 1 and 3 in Bz and Bt, and 1 and 4 in Q.

The Bz and Bt compounds could theoretically compete with purines for nucleoside formation by virtue of the similarity between nitrogen atom 1 and the pyridine nitrogen atom in purine position 9, and, indeed, Woolley (1944) and Kidder *et al.* (1949) have provided support for such an hypothesis by reversing the toxicity of several substituted benzimidazoles with purines in bacteria and

protozoa. However, reversal has not been observed in metazoa, including frog embryos (Manikis, 1959) and chick embryos (Billet & Perry, 1957*a*, *b*).



BENZIMIDAZOLE



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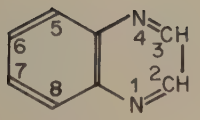
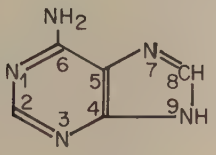
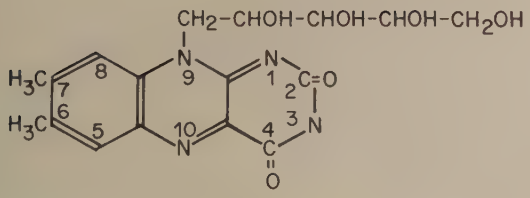


QUINOXALINE



ADENINE



RIBOFLAVIN

TEXT-FIG. 1. Structural formulae of Bz, Bt, and Q, a representative purine, and riboflavin. (*Chemical Abstracts* numbering system.)

It is usual for the strongly negative nitro or chloro loadings to enhance toxicity in compounds of this type. This was also observed in Arbacia eggs where chloro substitution in caffein was more inhibitory than ethoxy or methyl substitution (Cheney, 1957). The introduction of a carboxyl group into heterocyclic nitrogen compounds almost invariably diminishes toxicity, as it did decisively in our experiments, except for 2,3-diPh-7-N-5-Cx-quinoxaline.

The quinoxalines, on the other hand, can be likened to riboflavin, and conceivably compete with it for the formation of flavin nucleotides, which are presumably essential for development. We have not, however, carried out reversal experiments to test this possibility. It would not be anticipated that substitution in the benzene moiety would have much effect on the reactivity of quinoxaline while 2,3-substitution should decrease reactivity. It does so in our experiments at stage 3, but not at the older stages (Table 1). However, the reverse is true for 2,3-diMe-6-MO-5-N-Q, which is toxic for the embryos, while 6-MO-5-N-Q permits normal development. This latter compound illustrates

also the effect of position; 6-MO-5-N-Q is non-toxic, in contrast to 7-MO-5-N-Q and 5-MO-7-N-Q, both of which are toxic, as previously reported (Liedke, Engelman, & Graff, 1957*a*, *b*).

Assessment of the relation of structure to biological activity is complicated further by unanswered questions on solubility and transfer to the interior of the treated structures wherein metabolic activities are compartmentalized. The purines, riboflavins, folic acid, and vitamin B_{12} all play a role in development, but their concentrations, cellular sites, and rates of use are unknown and cannot be related to specific morphological events.

It has been observed in disrupted staphylococcus cells, on the other hand, that derivatives of benzimidazole in which the 5,6 positions are substituted by CH_3, Cl, or NO are potent inhibitors of glycine incorporation, but that 6-amino-4-hydroxy benzimidazole is an activator, suggesting that the actions of the inhibitory derivatives are due to structural analogies with benzimidazoles rather than with purines (E. F. Gale, personal communication).

SUMMARY

The effects of variously substituted quinoxalines, benzimidazoles, and benzotriazoles on *Rana pipiens* embryos of 2-cell (S. 3), blastula (S. 8), neurula (S. 14) and tail-bud stage (S. 18) were studied. It was found that:

1. Toxicity is variably increased by double substitution with two nitro groups (diN) or a nitro group and a chlorine atom (N+Cl). The relative toxicity of the single N-substituent is diminished by the addition of a hydroxy group, and generally also by a methoxy group, but not by addition of a methyl group; still greater is the diminishing effect of the carboxyl group which nullifies all activity. Cl-substitution in place of N is less toxic except at stages 3 and 8 with benzimidazoles.

2. Susceptibility to benzotriazoles increases with age of embryo from 2-cell to tail-bud stage 18, while the reverse is true for quinoxalines and benzimidazoles. The diN- or N+Cl-substituted compounds are exceptions, as they are highly toxic at all developmental stages under the experimental conditions.

3. In young tadpoles (S. 25) N-substitution increases the toxicity of quinoxaline or benzimidazole approximately 10 times, and of benzotriazole as much as 40 times.

RÉSUMÉ

Réactions de l'embryon à des inhibiteurs ayant une parenté structurale II. Les effets des substituants

Les effets de substituants variés des quinoxalines, des benzimidazoles, des benzotriazoles ont été étudiés sur les embryons de *Rana pipiens* aux stades 2 blastomères (St. 3), blastula (St. 8), neurula (St. 14) et bourgeon caudal (St. 18). Les résultats ont été les suivants:

1. La toxicité s'accroît d'une manière variable par la double substitution

avec 2 groupes nitro (di-N) ou un groupe nitro et un atome de chlore (N+Cl). La toxicité relative du simple substituant N est diminué par l'addition d'un groupe hydroxyl, et généralement aussi par un groupe méthoxyl; la diminution de l'effet toxique est encore plus importante avec le groupe carboxyl, qui annulle toute activité. La substitution de Cl à l'emplacement d'un N est moins toxique, sauf aux stades 3 et 8, dans le cas des benzimidazoles.

2. La sensibilité aux benzotriazoles s'accroît avec l'âge de l'embryon du stade 2 blastomères au stade du bourgeon caudal (St. 18), tandis que l'inverse est vrai pour les quinoxalines et les benzimidazoles. Les composés substitués par un di-N ou par N+Cl sont des exceptions, car ils sont extrêmement toxiques à tous les stades du développement dans les conditions expérimentales.

3. Chez les jeunes têtards (St. 25), la substitution de N augmente la toxicité des quinoxalines et des benzimidazoles d'environ 10 fois, celle des benzotriazoles d'au moins 40 fois.

REFERENCES

Bahner, G. T., Rutter, H. A., & Rives, L. M. (1952). Substituted benzimidazoles. *J. Amer. chem. Soc.* **74,** 3689.

Bamberger, E. (1893). Studien ueber Imidazole. *Liebigs Ann.* **273,** 340.

Billet, F., & Perry, M. M. (1957*a*). The effect of certain alkyl derivatives of benzimidazole on the embryos of *Xenopus laevis*. *Proc. R. phys. Soc. Edinb.* **25,** 59–62.

—— —— (1957*b*). The effect of certain alkyl derivatives of benzimidazole on the development of chick embryos grown *in vitro*. *Proc. R. phys. Soc. Edinb.* **26,** 15–18.

Bost, R. W., & Towell, E. E. (1948). The preparation of some substituted quinoxalines. *J. Amer. chem. Soc.* **70,** 903.

Cheney, R. H. (1957). Dioxypurine derivatives as mitotic and growth inhibitors. *Biol. Bull. Wood's Hole,* **113,** 239.

Fischer, O. (1904). Ueber Benzimidazole und Oxydationsprodukte von Orthodiaminen. *Ber. dtsch. chem. Ges.* **37,** 555.

Gillespie, H. B., Engelman, M., Spano, F., & Graff, S. (1957). Some new *o*-phenylenediamines and the related benzimidazoles, benzotriazoles, and quinoxalines. *J. Amer. chem. Soc.* **79,** 2245.

—— Spano, F., & Graff, S. (1960). Synthesis of some substituted benzimidazoles, benzotriazoles, and quinoxalines. *J. org. Chem.* In press.

Hofmann, A. W. (1860). Einwirkung der salpetrigen Saeure auf das Nitrophenylenediamine. *Liebigs Ann.* **115,** 251.

Hoover, J. R. E., & Day, A. R. (1955). Metabolic analogs. III. Preparation of some benzimidazoles with substituents on the 4(7)- and 6(5)-positions. *J. Amer. chem. Soc.* **77,** 4324.

Kidder, G. W., Parks, R. E., & Woodside, G. L. (1949). Purine metabolism in Tetrahymena and its relation to malignant cells in mice. *Science,* **109,** 511.

Liedke, K. B., Engelman, M., & Graff, S. (1954). The selective response of amphibian embryos to benzimidazole and benzotriazole derivatives. *J. exp. Zool.* **127,** 201–20.

—— —— —— (1955). Inhibitors of amphibian development. *Anat. Rec.* **123,** 359–84.

—— —— —— (1957*a*). Specificity of embryonic response to chemical inhibitors. *J. exp. Zool.* **136,** 117–42.

—— —— —— (1957*b*). Embryonic responses to structurally related inhibitors. *J. Embryol. exp. Morph.* **5,** 368–76.

Manikis, L. A. (1959). Alteration of growth and differentiation of *Rana* embryos by benzimidazole. *Natural appl. Sci. Bull.* **16,** 137–78.

Nietzki, R., & Hagenbach, H. (1897). Die Reduktion des Pikramids. *Ber. dtsch. chem. Ges.* **30,** 544.

Shumway, W. (1940). Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.* **78,** 139–47.

Ullmann, F., & Mauthner, F. (1903). Ueber die Oxydation von substituierten *o*-Phenylenediaminen. *Ber. dtsch. chem. Ges.* **36,** 4028.

Woolley, D. W. (1943). Some biological effects produced by benzimidazole and their reversal by purines. *J. biol. Chem.* **152,** 225–32.
—— (1944). *A Study of Antimetabolites.* New York: John Wiley & Sons Inc.

EXPLANATION OF PLATES

PLATE 1

Figs. A–D. Photographs of transverse sections through the eye and forebrain region of 11-mm. experimental tadpoles. Magnifications ×100, except for fig. C (×400).

Fig. A. After 3 days in 20 μg./ml. benzotriazole (Bt). Note massive necrosis in ventral portion of brain, isolated pycnotic nuclei (*n*) in retina and cartilage, and simultaneous circulation failure, i.e. blood-vessels obliterated and lens pressed into eye-cup. Liver necrosis seen in fig. F.

Fig. B. After 2 days in 20 μg./ml. N-Bz. Note necrosis of proliferating and early differentiating cells in iris angle, pycnotic nuclei in brain, no disturbance in circulation except for some necrotic cells in liver sinuses shown in fig. G.

Fig. C. After 1 day in 20 μg./ml. N-Q. Note only a few pycnotic cells (*n*) in iris angle; normal circulation and liver, except for choroid plexus shown in fig. H.

Fig. D. After 1 day, following 70 minutes exposure to 20 μg./ml. N-Bt. Note that absorption of necrotic cells in the thinner retina and the brain is still incomplete; normal blood-cells and liver (fig. E).

PLATE 2

Figs. E–H. Photographs of transverse sections through the liver or forebrain of the same experimental tadpoles as shown in figs. A–D. Magnifications ×300, except for fig. F (×100).

Fig. E. After 1 day, following 70 minutes exposure to 20 μg./ml. N-Bt. Note active mitoses of blood-cells in liver and normal pronephric tubules.

Fig. F. After 3 days in 20 μg./ml. Bt. Note complete circulation failure, liver necrosis, and distension of pronephric tubules.

Fig. G. After 2 days in 20 μg./ml. N-Bz. Note pycnotic and normal blood-cells in liver sinuses.

Fig. H. After 1 day in 20 μg./ml. N-Q. Note necrosis of blood-cells (*nB*) in choroid plexus and contraction of pigment cells (*p*).

Abbreviations: *h* = hypertrophied cell; *n* = necrotic cell; *nB* = necrotic blood-cell; *m* = mitotic cell; *p* = pigment cell; *Pro* = Pronephros.

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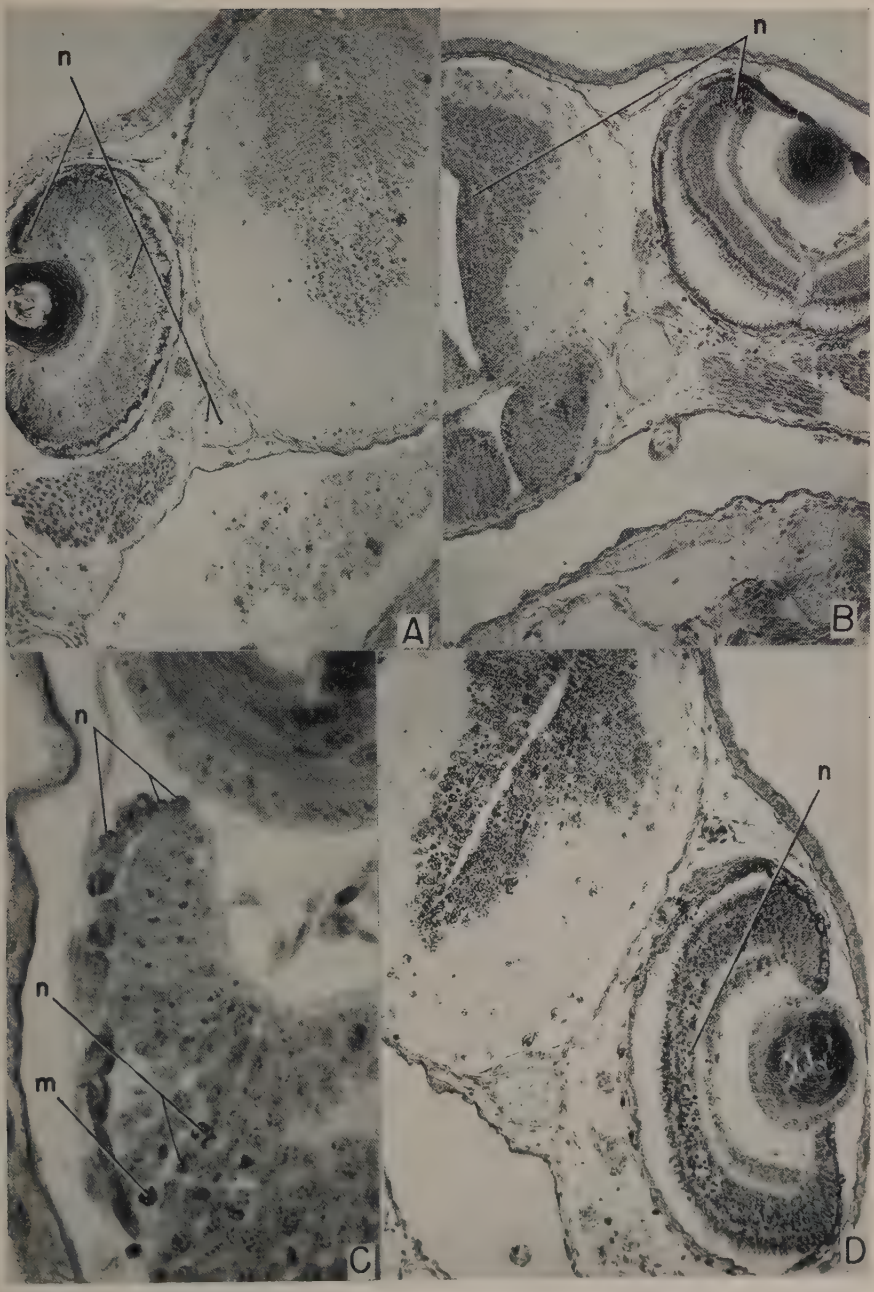

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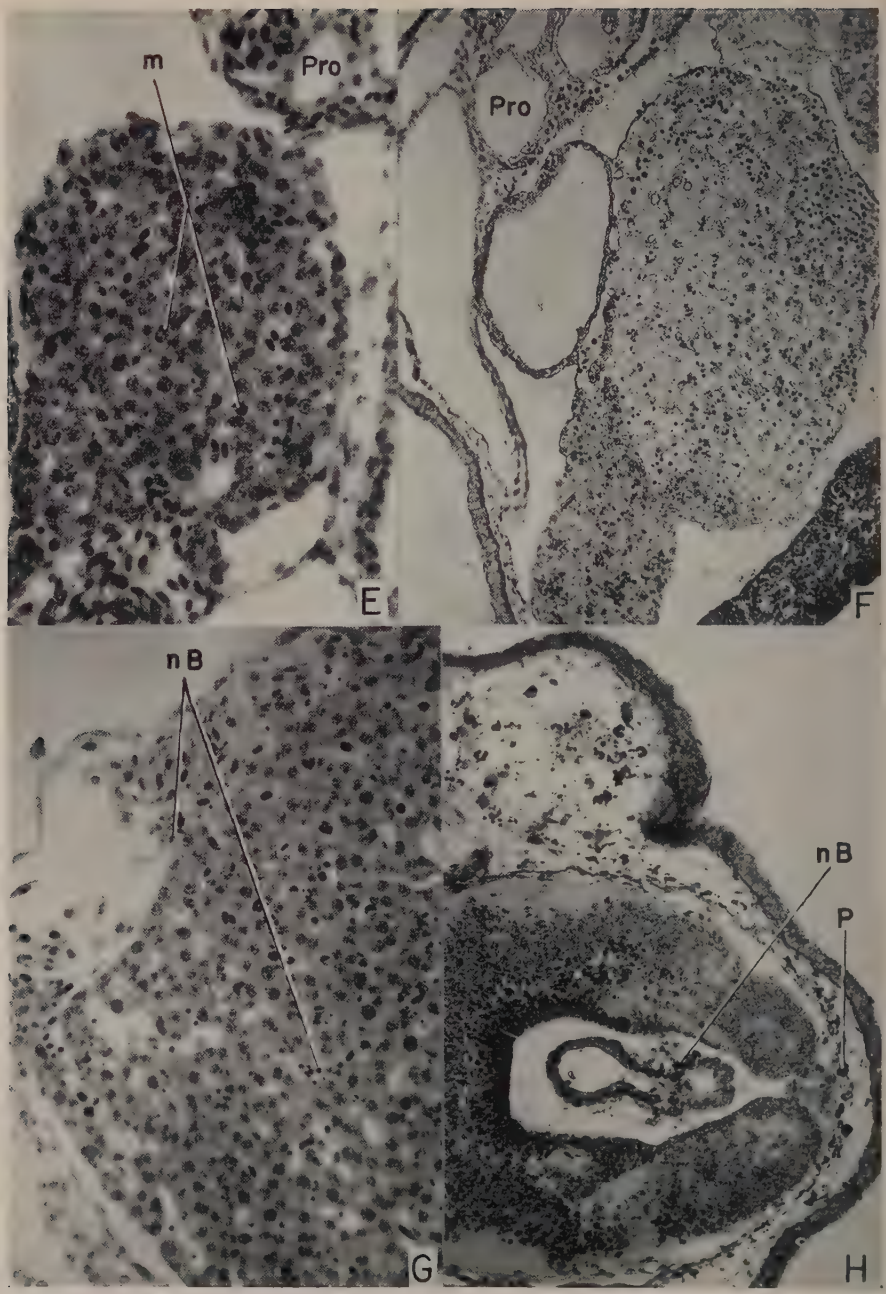
Plate 1



K. LIEDKE, H. B. GILLESPIE, *and* S. GRAFF

Plate 2

Rôle inducteur du mésoderme dans la différenciation précoce du bourgeon de membre chez l'embryon de Poulet

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AVEC DEUX PLANCHES

INTRODUCTION

Le bourgeon de membre des Vertébrés est composé de deux ébauches, l'une ectodermique, l'autre mésodermique provenant d'un épaississement de la somatopleure.

Quel est, de ces deux composants, celui qui déclenche l'excroissance du bourgeon et joue le rôle de l'inducteur primaire du membre?

Cette question a été résolue chez les Amphibiens Urodèles. Les expériences de Harrison (1925, 1931), de Balinsky (1931) et de Rotmann (1931) ont démontré l'action inductrice du mésoderme sur l'ectoderme sus-jacent lors de l'apparition du bourgeon de membre. Récemment, Tschumi (1957) a démontré les mêmes propriétés du mésoderme chez un Anoure (*Xenopus*).

Le mésoderme du bourgeon de membre des Oiseaux possède-t-il ce même pouvoir inducteur?

On a beaucoup écrit sur les rôles respectifs du mésoderme et de l'ectoderme dans la différenciation du membre. Rappelons simplement, parmi de nombreux travaux, les recherches de Saunders (1948, 1949), Zwilling (1955, 1956) et surtout de Hampé (1957, 1959) qui ont mis en évidence le rôle actif de l'ectoderme dans la croissance et dans la différenciation des articles du membre. D'autre part, le mésoderme semble jouer un rôle non moins important puisque c'est lui qui détermine la qualité (aile ou patte) de cette différenciation. Pourtant la question de la détermination précoce et de l'induction primaire du bourgeon de membre n'a pas encore été résolue.

Les auteurs, qui jusqu'ici ont étudié le rôle du mésoderme, se sont adressés au bourgeon de membre déjà formé, tel qu'il se présente à partir du stade 18 de Hamburger & Hamilton (1951). Les résultats qu'ils ont obtenus sont contradictoires. D'une part, Zwilling (1955) montre que le mésoderme, séparé de l'ectoderme par le versène et implanté sous l'ectoderme banal situé en dehors du

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territoire du membre est incapable de provoquer la différenciation d'un membre. D'autre part, Bell, Kaighn & Fessenden (1959) ont montré, tout récemment, que le mésoderme du bourgeon de membre séparé de l'ectoderme par les ultrasons et implanté en greffe coelomique est capable de se différencier en articles cartilagineux et en musculature, même en l'absence de tout revêtement ectodermique. Ce résultat met en doute le rôle actif de la cape apicale ectodermique et se trouve ainsi être en contradiction avec les conclusions des auteurs cités plus haut. Quelles que soient les interactions entre le mésoderme et l'ectoderme au cours de la différenciation du membre, nous avons voulu préciser, par de nouvelles interventions pratiquées à un stade plus jeune, le rôle du mésoderme dans la détermination précoce du membre (Kieny, 1959).

MATÉRIEL ET MÉTHODES

Les expériences ont été faites sur des embryons de Poulet de la race Leghorn blanche.

Du mésoderme présomptif de membre ou du mésoderme de jeune membre est greffé dans le flanc d'un embryon, dans la région située entre l'aile et la patte. Le stade des embryons donneurs de mésoderme varie du stade 15 à 16 au stade 18 de Hamburger & Hamilton. Les embryons hôtes possèdent de 15 à 23 somites (stades 12 à 14).

ISOLEMENT DU MÉSODERME

Nous nous sommes servis de deux agents de dissociation, la trypsine et le versène.

Dissociation à l'aide de la trypsine

Nous avons utilisé la méthode à la trypsine diluée élaborée par Moscona (1952). L'ensemble ectoderme-mésoderme séjourne dans une solution de trypsine Difco 1 : 250 à 2 pour cent dans du liquide de Tyrode, pendant 2 à 5 minutes à 38° C. (Planche 1, fig. 2). Après plusieurs bains de lavage dans du liquide de Tyrode, on détache facilement l'ectoderme du mésoderme à l'aide de deux microscalpels.

Dissociation à l'aide du versène (versenate de Na)

La plus grande partie des bourgeons de membre a été soumise à l'action du versène. La technique employée est celle qui a été mise au point par Zwilling (1955) pour la séparation de l'ectoderme et du mésoderme: action d'une solution de versène dans du liquide de Tyrode dépourvu de Ca et de Mg pendant 20 à 30 minutes à 38° C. (Planche 1, fig. 1).

Dilacération du mésoderme

Dans quelques expériences le bain dans la solution de trypsine a été prolongé jusqu'à 30 minutes, le bain dans la solution de versène jusqu'à 60 à 70 minutes.

On détache l'ectoderme après 2 à 5 minutes dans le cas de la trypsine et après 20 à 30 minutes dans le cas du versène. Après plusieurs bains de lavage, le mésoderme est déchiqueté en une douzaine de petits fragments. Après quelques minutes les fragments réagglomérés sont greffés de la manière habituelle.

Greffe du mésoderme dans le flanc d'un embryon

Le mésoderme obtenu après action de la trypsine ou du versène est greffé dans l'aire pellucide d'un embryon de 15 à 23 somites à égale distance des futures ébauches de l'aile et de la patte. Pour les stades les plus jeunes (antérieurs à 20 somites) on définit la région du flanc d'après la carte des territoires présomptifs de l'aile et de la patte mise au point par Chaube (1959). On pratique une fente perpendiculairement à l'axe de l'embryon à l'aide d'une aiguille de verre. On y introduit le greffon de sorte que $\frac{1}{3}$ environ du mésoderme émerge. Ainsi le mésoderme se trouve mis en contact avec la région de la somatopleure destinée normalement à former le flanc de l'embryon. Les hôtes sont sacrifiés au 11ème jour d'incubation. Le squelette est mis en évidence par la technique de Lundvall.

Greffe du mésoderme sur la membrane chorio-allantoïdienne

Du mésoderme de membre, après action du versène, a été greffé sur la membrane chorio-allantoïdienne d'embryons de 6 à 7 jours $\frac{1}{2}$ d'incubation. Après 7 jours, le greffon a été fixé. Le développement cartilagineux a été mis en évidence selon la technique de Lundvall.

Greffe de l'ectoderme apical du membre induit sur un moignon de patte

On prélève la partie distale ou la totalité du bourgeon de membre surnuméraire induit, 30 à 40 heures après l'implantation du mésoderme. L'ectoderme est séparé du mésoderme par la technique de la trypsine diluée (voir p. 458). Le revêtement ectodermique est greffé sur un moignon de patte sectionné au niveau du genou. Cet ectoderme est maintenu en place à l'aide de deux fins crochets d'argent.

Les embryons donneurs, ayant fourni l'ectoderme apical du membre surnuméraire, ainsi que les embryons hôtes, qui ont reçu la greffe d'ectoderme, sont sacrifiés au 11ème jour d'incubation pour permettre la mise en évidence du squelette cartilagineux.

Techniques histologiques

Pour l'étude histologique, des greffons ont été inclus à la paraffine, coupés à 5 microns et colorés au glychémalun-éosine.

INDUCTION D'UN MEMBRE SURNUMÉRAIRE

Greffe de mésoderme de membre soumis à l'action de la trypsine

Dans nos premières expériences nous avons utilisé la méthode de la trypsine diluée (Moscona, 1952) pour séparer le mésoderme et l'ectoderme du bourgeon de membre. Le mésoderme de l'ébauche présumée du bourgeon de membre (stades 16 à 17) ou le mésoderme du jeune bourgeon de membre (stades 17 à 18) a été greffé dans le flanc d'embryons de 15 à 23 somites à égale distance entre la future aile et la future patte. Les résultats globaux de cette série expérimentale sont consignés dans le tableau 1. Sur les 59 embryons hôtes, qui ont vécu au-delà

TABLEAU 1

Greffe de mésoderme de membre après action de la trypsine

	Nombre de cas
Nombre d'embryons hôtes	103
Nombre d'embryons morts avant 7 j. d'incubation	44
Différenciation de parties distales (tendance à la formation d'un membre)	1
Différenciation de masses cartilagineuses selon la valeur prospective du greffon	10
Greffon non retrouvé	48

de 7 jours et qui permettent une bonne mise en évidence du squelette, 1 seul embryon présente une formation surnuméraire de type membre (Planche 1, fig. 3). Sur les 58 embryons restants, 22 embryons présentent, à l'emplacement de la greffe, une plage de germes plumaires bien développés et spécifiques du bassin ou du stylopode, bien que le flanc de l'hôte soit normalement dépourvu de germes plumaires à ce stade. La peau du flanc est capable de répondre à une induction du mésoderme de membre. De ces 22 embryons, seuls 10 présentent une formation cartilagineuse conforme à la valeur prospective du greffon. Cette différenciation cartilagineuse peut être plus ou moins importante. Elle peut se réduire à un minuscule osselet informe, ou par contre, elle peut représenter une partie de l'os stylopodial; dans les cas les mieux réussis le greffon provenait d'embryons de stade 18.

Greffe de mésoderme soumis à l'action du versène

Après avoir obtenu uniquement des résultats négatifs avec le mésoderme soumis à l'action de la trypsine, nous nous sommes servis de la méthode de dissociation au versène préconisée par Zwilling (1955). Le stade des embryons donneurs de mésoderme varie du stade 15–16 au stade 17–18,[1] celui des embryons hôtes du stade 12 au stade 14 (44 heures d'incubation: 15 à 23 somites). Les résultats de cette série expérimentale sont résumés dans le tableau 2. Parmi les 54 embryons hôtes, qui ont vécu au-delà de 7 jours, 22 ont développé un

[1] Des expériences en cours ont permis de montrer l'activité inductrice du mésoderme de la future ébauche de membre à partir du stade 20 somites.

membre surnuméraire bien formé (Planche 1, figs. 4, 5, 6, 7). Vingt-cinq des embryons restants présentent une formation cartilagineuse qui correspond simplement à la valeur prospective du greffon (Planche 1, fig. 8). Enfin, chez 7 embryons, le greffon n'a pas été retrouvé et l'hôte ne montre aucune différenciation cartilagineuse supplémentaire. Signalons cependant que 10 des 32

TABLEAU 2

Greffe de mésoderme de membre après action du versène

	Stade du mésoderme greffé					
	< 16	*16*	*16–17*	*17*	*17–18*	*Total*
Nombre d'embryons morts avant le 8ème jour d'incubation	7	2	4	9	7	29
Formation de membre surnuméraire	11	4	1	3	3	22
Différenciation de masses cartilagineuses selon la valeur prospective du greffon	..	1	4	10	10	25
Greffon non retrouvé	..	1	1	1	4	7
TOTAL	18	8	10	23	24	83

cas négatifs présentaient, 40 heures après l'implantation, des ébauches de membre surnuméraire, dont la palette était nantie de la vascularisation typique du bourgeon de membre de 3 jours. Rien, dans leur structure macroscopique, ne permettait de prévoir que ces différenciations régresseraient par la suite. Dans 8 cas, nous n'avons retrouvé qu'une formation cartilagineuse correspondant à la valeur prospective du greffon. Dans 2 cas l'involution a été totale.

Les 22 membres surnuméraires bien formés se sont différenciés selon la nature d'aile ou de patte du mésoderme implanté (3 ailes et 19 pattes). Tous les membres sont entièrement recouverts d'ectoderme normalement différencié en germes plumaires et en écailles.

TABLEAU 3

Différenciation autopodiale des membres surnuméraires

Nature du greffon	*Nombre de membres surnuméraires qui ont formé*				
	1 doigt	*2 doigts*	*3 doigts*	*4 doigts*	*plus de 4 doigts*
Patte	3	9	4	1	2
Aile	2	1	..	..	..

Dans la majorité des cas, le membre surnuméraire possède 3 articles. Douze membres de type patte sont particulièrement bien constitués et les différentes pièces osseuses sont facilement identifiables. Lorsque le nombre des articles est inférieur à 3 (3 cas), l'autopode pourtant ne fait jamais défaut. Le nombre des rayons varie de 1 à 6 avec une forte proportion de pattes à 2 rayons (tableau 3).

Le membre surnuméraire est toujours orienté vers l'extérieur (Planche 1, figs. 4, 5, 6, 7). Il se trouve placé dans le même plan que les membres de l'hôte,

malgré l'orientation quelconque du greffon. Il semble donc que le mésoderme, au stade où il est prélevé, ne possède pas encore de polarité bien établie. Comme le membre se différencie toujours en direction de l'ectoderme qui le recouvre, il est possible que l'ectoderme joue un rôle déterminant dans la direction de croissance du membre. Cette opinion est confirmée par les résultats de la série expérimentale suivante.

Greffe de mésoderme dilacéré de membre

Le mésoderme dilacéré après l'action du versène conserve tout son pouvoir inducteur. On a obtenu la différenciation d'un membre surnuméraire dans la moitié des cas (Planche 2, figs. 9, 10; tableau 4). Leur orientation est la même que dans la série précédente. Il est donc raisonnable de penser que le rôle orienteur du membre revient à l'ectoderme.

Après l'action de la trypsine, le mésoderme dilacéré n'a donné aucun résultat positif.

TABLEAU 4

Greffe de mésoderme après action prolongée du versène et dilacération mécanique

Stade du mésoderme prélevé:	*< 16*	*16*	*16–17*	*17*	*17–18*	*Total*
Nombre d'embryons morts avant le 8ème jour d'incubation	1	..	1	1	..	3
Formation d'un membre surnuméraire	1	1	2	4	1	9
Greffon non retrouvé	1	1	..	4	2	8

Greffes témoins de mésoderme de membre sur la membrane chorio-allantoïdienne

Du mésoderme de membre de stades 15–16 à 19, après l'action du versène, a été greffé sur la membrane chorio-allantoïdienne. Il évolue, dans la moitié des cas, en formant des masses cartilagineuses globuleuses et morphologiquement indifférenciées (Planche 2, fig. 11; tableau 5).

TABLEAU 5

Évolution du mésoderme greffé sur la membrane chorio-allantoïdienne

Stade du prélèvement: / *Évolution du greffon*	*< 16*	*16*	*16–17*	*17*	*17–18*	*18*	*18–19*	*19*	*Total*
Vésicule contenant des différenciations cartilagineuses	..	..	2	..	11	6	4	3	26
Vésicule vide	1	..	..	1	..	..	..	..	2
Greffon non retrouvé	5	4	2	3	5	3	..	2	24

Les bourgeons de membre témoins de cette série expérimentale, qui ont été traités au versène, mais dont l'ectoderme est laissé en place, forment des membres presque complets, lorsqu'ils sont greffés sur la membrane chorio-allantoïdienne (Planche 2, fig. 12).

Conclusions

Le mésoderme d'un jeune bourgeon de membre ou le mésoderme présomptif du membre est capable d'induire un membre surnuméraire, lorsqu'il est greffé sous l'ectoderme banal compris entre l'aile et la patte. L'ectoderme, sous l'influence inductrice du mésoderme greffé, participe à l'édification du membre. Nous pouvons donc conclure que le mésoderme est l'inducteur primaire du membre.

Il est important de noter que le mésoderme n'est capable d'exercer son activité organisatrice que s'il a été décortiqué après l'action du versène. Après le passage dans la solution de trypsine, le greffon ne se différencie guère et ne peut donner naissance à un membre surnuméraire.

MÉCANISME DU DÉVELOPPEMENT DU MEMBRE SURNUMÉRAIRE

Les expériences décrites démontrent nettement que le mésoderme est l'inducteur primaire du bourgeon de membre. Le développement de ce membre surnuméraire se fait-il comme celui du membre normal? En d'autres termes, le mésoderme greffé en dehors du territoire du membre, induit-il dans l'ectoderme banal du flanc la formation d'une crête apicale ectodermique?

Pour répondre à cette question il suffisait de montrer que le revêtement ectodermique apical du bourgeon de membre surnuméraire possède les mêmes propriétés inductrices sur le mésoderme que la calotte apicale du bourgeon de membre normal.

Nous avons copié une des expériences particulièrement nettes de Hampé (1959): il greffe une calotte apicale d'aile ou de patte de stade 18 à 26 sur un moignon de patte de stade 20 sectionné au niveau du genou. Dans ces conditions, il obtient un membre plus ou moins complet suivant le stade de la calotte apicale greffée. Ce membre comporte toujours des orteils, souvent un tibia, un péroné et des métatarses.

L'extrémité distale du membre induit est constituée d'un bourrelet marginal ectodermique et offre l'aspect d'une véritable calotte apicale.

Le revêtement ectodermique apical du membre surnuméraire est greffé sur un moignon de patte d'un autre embryon de stade 19 à 20. Le membre surnuméraire est amputé 30 à 40 heures après la greffe du mésoderme dans le flanc, c'est-à-dire approximativement au stade 20. A ce moment, l'embryon porteur du membre surnuméraire a atteint le stade 19 à 24.

Dans tous les cas, après l'amputation de sa partie distale, le membre surnuméraire s'arrête de croître et ne différencie jamais son squelette distal (Planche 2, fig. 13). Trente-quatre greffes ont été réalisées. Vingt-six embryons ont vécu au-delà du 8ème jour d'incubation et 6 d'entre eux présentent sur le membre opéré des différenciations distales de type patte (fig. 1; Planche 2, fig. 14). Dans

4 cas, la patte reconstituée possède 3 articles. Dans les 2 autres cas, l'orteil qui s'est développé n'est pas raccordé au squelette axial.

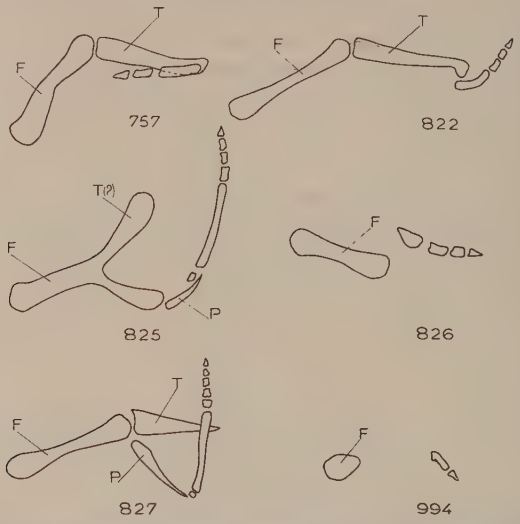

FIG. 1. Greffe de la calotte apicale du membre surnuméraire sur un moignon de patte sectionné au niveau du genou. Représentation schématique des membres qui se sont développés. La greffe d'un ectoderme prélevé sur le flanc d'un embryon comme celle d'un ectoderme prélevé sur la face dorsale ou ventrale d'un bourgeon de membre (Hampé, 1957) ne permet en aucun cas la différenciation distale du moignon.

Ces expériences démontrent nettement que les propriétés du revêtement ectodermique du membre surnuméraire sont équivalentes à celles de la calotte apicale d'un membre normal.

Nous pouvons donc conclure que le mésoderme greffé en dehors du territoire du membre, induit dans l'ectoderme banal du flanc, la formation d'une calotte apicale active.

CONCLUSIONS GÉNÉRALES

Nous avons greffé du mésoderme du territoire présomptif du membre (stade 15 à 16 jusqu'au stade 17 à 18) sous l'ectoderme latéral d'embryons de 15 à 23 somites. Le greffon est implanté entre les futures ébauches de l'aile et de la patte.

Dans ces conditions, environ 40 pour cent des greffons mésodermiques provoquent le développement d'un membre en induisant dans l'ectoderme la formation d'une calotte apicale. Cette calotte apicale a les mêmes propriétés inductrices sur le mésoderme sous-jacent que la calotte apicale d'un membre normal. La différenciation des articles distaux ne se réalise pas en l'absence d'ectoderme. De plus, la calotte apicale est capable d'induire dans un moignon stylopodial la formation d'un membre à 3 articles.

Les résultats négatifs de Zwilling nous font penser qu'au stade où il opère (72 heures) l'ectoderme a peut-être perdu la capacité de répondre à l'induction mésodermique.

Nous pouvons donc conclure:

(1) que l'ectoderme situé en dehors du territoire membre peut contribuer à l'édification d'un membre, lorsqu'il a été soumis à l'action inductrice primaire du mésoderme de membre;

(2) que l'ectoderme réagit en formant une calotte apicale, qui, à son tour, exerce une action inductrice sur le mésoderme sous-jacent.

RÉSUMÉ

1. Du mésoderme de l'ébauche présumée du bourgeon de membre (stades 15 à 16 et 17 de Hamburger & Hamilton) ou du mésoderme d'un très jeune bourgeon de membre (stades 17 à 18) est séparé de l'ectoderme après action de la trypsine ou du versène et greffé dans l'aire pellucide d'un embryon de 15 à 23 somites (stades 12 à 14). Le mésoderme se trouve en contact avec la région de la somatopleure destinée normalement à former le flanc de l'embryon compris entre l'aile et la patte.

2. Le mésoderme, après passage dans la solution de trypsine, a provoqué la formation d'un membre surnuméraire dans 1 cas sur 59 seulement (Planche 1, fig. 3).

3. Après l'action du versène, le mésoderme implanté induit la formation d'un membre surnuméraire dans 22 cas sur 54 (Planche 1, figs. 4, 5, 6, 7). Même après dilacération, le mésoderme induit encore des membres surnuméraires (Planche 2, figs. 9, 10). Tous ces membres surnuméraires sont recouverts d'épiderme, poussent vers l'extérieur et se trouvent placés dans le même plan que les membres de l'embryon hôte.

4. Des greffons témoins de mésoderme sur la membrane chorio-allantoïdienne forment quelques amas cartilagineux informes; leur différenciation n'est en aucun cas supérieure à la valeur prospective du mésoderme greffé (Planche 2, fig. 11).

5. L'ectoderme du flanc, sous l'influence inductrice du mésoderme, participe à l'édification du membre surnuméraire en différenciant une calotte apicale qui possède les mêmes propriétés que la calotte apicale d'un membre normal (fig. 1; Planche 2, fig. 14).

6. Le mésoderme de l'ébauche de membre est l'inducteur primaire du membre.

SUMMARY

1. Mesoderm from the presumptive anlage of the limb-bud (stages 15–16 and 17 of Hamburger & Hamilton) or mesoderm from a very young limb-bud (stages 17–18) was separated from its ectoderm with trypsin or versene and grafted into the area pellucida of an embryo of 15–23 somites (stages 12–14). The mesoderm was placed in contact with the region of the somatopleure which normally gives rise to the flank of the embryo between the wing and the leg.

2. After the action of the trypsin solution, the mesoderm elicited the outgrowth of a supernumerary limb in only one case out of 59 (Plate 1, fig. 3).

3. After the action of the versene, the implanted mesoderm induced an extra limb in 22 cases out of 54 (Plate 1, figs. 4, 5, 6, 7). The inductive capacity was retained after the mesoderm had been chopped to small pieces before grafting (Plate 2, figs. 9, 10). All induced limbs grew outwards and were situated in the same plane as the limbs of the host embryo. They were all covered by host epidermis.

4. Control grafts of mesoderm on chorio-allantoic membrane only formed amorphous cartilaginous masses; their development was in no case greater than their prospective value (Plate 2, fig. 11).

5. The host flank ectoderm, under the influence of the grafted mesoderm, formed an ectodermal ridge possessing the same properties as the ectodermal ridge of a normal limb-bud (text-fig. 1; Plate 2, fig. 14).

6. It is concluded that the limb-bud mesoderm is the primary inductor of the limb.

TRAVAUX CITÉS

Balinsky, B. I. (1931). Zur Dynamik der Extremitätenknospenbildung. *Roux Arch. EntwMech. Organ.* **123,** 565–632.

Bell, E., Kaighn, M. E., & Fessenden, L. M. (1959). The role of mesodermal and ectodermal components in the development of the chick limb. *Devel. Biol.* **1,** 101–24.

Chaube, Sh. (1959). On axiation and symmetry in transplanted wing of the chick. *J. exp. Zool.* **140,** 29–78.

Hamburger, V., & Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88,** 49–92.

Harrison, R. G. (1925). The effect of reversing the medio-lateral or transverse axis of the forelimb bud in the salamander embryo. *Roux Arch. EntwMech. Organ.* **106,** 469–502.

—— (1931). Experiments on the development and growth of limbs in the Amphibia. *Science,* **74,** 575–6.

Hampé, A. (1957). Recherches sur la régulation des déficiences et des excédents du bourgeon de la patte du Poulet. *Arch. Anat. micr. Morph. exp.* **46,** 265–82.

—— (1959). Contribution à l'étude du développement et de la régulation des déficiences et des excédents dans la patte de l'embryon de Poulet. *Arch. Anat. micr. Morph. exp.* **48,** 345–478.

Kieny, M. (1959). Rôle du mésoderme dans le développement du bourgeon de membre chez l'embryon de Poulet. *C.R. Acad. Sci. Paris,* **249,** 1571–3.

Moscona, A. (1952). Cell suspensions from organ rudiments of chick embryo. *Exp. Cell. Res.* **3,** 535–9.

Rotmann, E. (1931). Die Rolle des Ektoderms und Mesoderms bei der Formbildung der Extremitäten von Triton. I. Operation in Gastrula-stadium. *Roux Arch. EntwMech. Organ.* **124,** 747–94.

Saunders, J. W. (1948). The proximo–distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. exp. Zool.* **108,** 363–404.

—— (1949). An analysis of the role of the apical ridge of ectoderm in the development of the limb bud in the chick. *Anat. Rec.* **105,** 567–8.

Tschumi, P. A. (1957). The growth of the hind limb bud of *Xenopus laevis* and its dependence upon the epidermis. *J. Anat. Lond.* **91,** 149–73.

Zwilling, E. (1955). Ectoderm–mesoderm relationship in the development of the chick embryo limb bud. *J. exp. Zool.* **128,** 423–41.

—— (1956). Reciprocal dependence of ectoderm and mesoderm during chick embryo limb development. *Amer. Nat.* **90,** 257–65.

EXPLICATION DES PLANCHES

Planche 1

Figs. 1, 2. Bourgeons de patte controlatéraux de stade 16 à 17, soumis d'une part (fig. 1) pendant 30 minutes à l'action du versène, d'autre part (fig. 2) pendant 2 minutes à l'action de la trypsine.

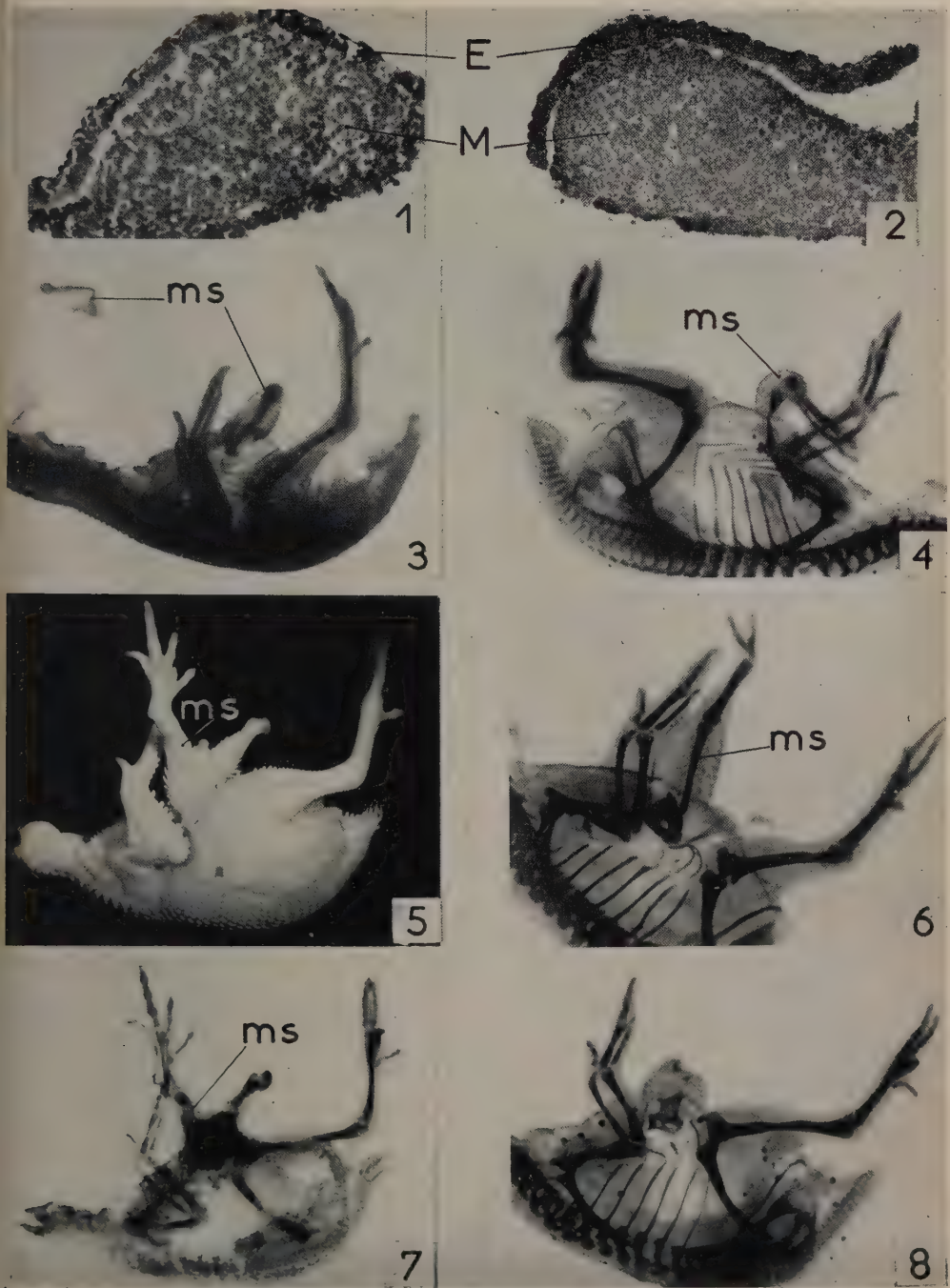

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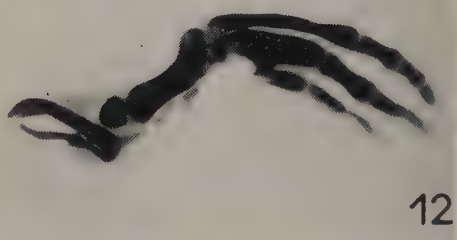
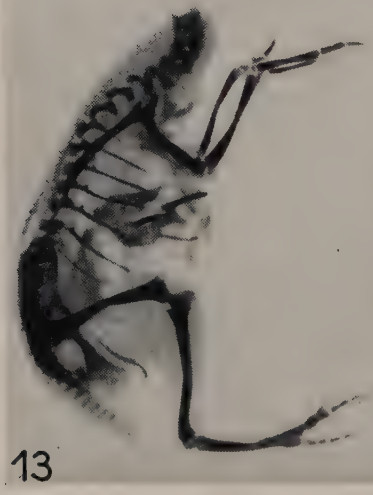
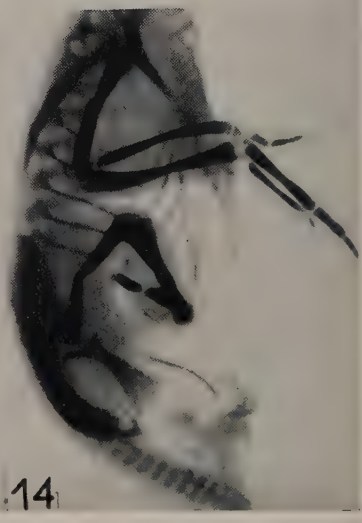
Planche 1







M. KIENY

Planche 2

Après l'action de la trypsine les cellules mésodermiques sont gonflées et serrées les unes contre les autres. Par contre, après l'action du versène, les cellules mésodermiques sont détachées les unes des autres. E, ectoderme; M, mésoderme; ×192.

FIG. 3. Le mésoderme d'un bourgeon de patte de stade 16, après action de la trypsine, a été greffé dans le flanc d'un embryon de 20 somites. L'extrémité distale du membre surnuméraire (*ms*), rattachée par un étranglement à la base du membre, s'est détachée au cours de la prise de vue. Elle figure dans le petit rectangle en haut à gauche.

FIG. 4. Mésoderme de bourgeon de patte de stade 16, après action du versène, greffé dans le flanc d'un embryon de 19 somites. Développement d'une patte à 2 articles (*ms*). On ne peut préciser si c'est le stylopode ou le zeugopode qui fait défaut.

FIGS. 5, 7. Mésoderme d'un bourgeon de patte de stade 16, après action du versène, greffé dans le flanc d'un embryon de 21 somites. Le membre surnuméraire (*ms*) est double. La base hémorragique renferme deux fémurs. La patte représentée entièrement contient un tibia trapu, elle est terminée par 4 doigts presque normaux. La patte repliée est formée d'un tibia moins court et des rayons I, II et III. Fig. 5: avant éclaircissement; fig. 7: après éclaircissement.

FIG. 6. Mésoderme de bourgeon de patte de stade 15 à 16, après action du versène, greffé dans le flanc d'un embryon de 20 somites. Développement d'une patte à 3 articles (*ms*).

FIG. 8. Mésoderme d'un bourgeon de patte de stade 17, après action du versène, greffé dans le flanc d'un embryon de 21 somites. Développement d'un os de la ceinture, selon la valeur prospective du greffon, dans une protubérance qui est recouverte de germes plumaires spécifiques de la hanche.

PLANCHE 2

FIGS. 9, 10. Mésoderme de bourgeon de patte de stade 17, greffé dans le flanc d'embryons de 19 somites. Après l'action du versène (1 heure), le mésoderme a été déchiqueté en petits fragments. Développement de membres surnuméraires (*ms*) identique à celui obtenu avec le mésoderme non dilacéré (comparer avec les figures 4, 5, 6, 7).

FIG. 11. Greffe chorio-allantoïdienne du mésoderme de l'ébauche présumée du bourgeon de patte d'un embryon de 29 somites. Formation d'une vésicule qui contient 3 nodules cartilagineux informes.

FIG. 12. Greffe chorio-allantoïdienne témoin. L'ébauche présumée du bourgeon de patte contro-latéral a été soumise à l'action du versène, mais l'ectoderme n'a pas été décollé. Développement d'une patte à 3 orteils. Le mésoderme pur de membre est incapable de former un membre lorsqu'il est greffé sur la membrane chorio-allantoïdienne.

FIG. 13. Bourgeon de membre surnuméraire dont on a amputé l'extrémité distale 40 heures après la greffe du mésoderme. On distingue un os de ceinture prolongé d'un os long. Les segments distaux du membre ne se sont pas développés.

FIG. 14. Le revêtement ectodermique apical d'un bourgeon de membre surnuméraire de stade 20 à 21, a été greffé sur un moignon de patte de stade 19 sectionné au niveau du genou. La patte qui s'est développée présente un fémur, un tibia, un métatarse prolongé de 2 phalanges.

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Culture *in vitro* de blastèmes de régénération de Planaires

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INTRODUCTION

Les problèmes de la régénération ont donné lieu à des recherches particulièrement approfondies chez les planaires, animaux dont le pouvoir de régénération est extraordinairement élevé. De nombreux auteurs ont étudié les modalités de la reconstitution, après une amputation, des divers organes et parties du corps de la planaire. De quel matériel cellulaire est constitué le jeune blastème de régénération? Quelle est l'origine, quelle est la nature de ces cellules? Wolff & Dubois, en 1947 et 1949, ont contribué à répondre à ces questions. Grâce à une méthode d'irradiations localisées aux rayons X, ces auteurs ont montré l'existence, chez les planaires, de cellules spéciales, migratrices, les néoblastes. Après une amputation, ces cellules affluent vers la surface de section et vont constituer le blastème de régénération. Les néoblastes sont des cellules indifférenciées, à gros noyau, au cytoplasme peu abondant. Elles présentent une grande activité mitotique, et sont totipotentes. Ces caractères sont ceux de cellules embryonnaires. Le jeune blastème peut donc être considéré comme une véritable ébauche embryonnaire, dont il nous a paru intéressant de connaître le destin lorsqu'elle est séparée de l'organisme qui lui a donné naissance: nous avons tenté de cultiver *in vitro* les blastèmes de régénération encore indifférenciés, en utilisant la méthode de culture mise au point par Wolff & Haffen (1952), à laquelle nous avons apporté certaines modifications.

MATÉRIEL ET TECHNIQUE

Toutes les expériences ont porté sur des animaux de l'espèce *Dugesia lugubris*.

Préparation des blastèmes utilisés pour la culture

Les planaires ont été opérées de manière à fournir des blastèmes de régénération de deux sortes: blastèmes antérieurs (futures têtes), d'une part; blastèmes postérieurs (futures queues), d'autre part.

Elles sont coupées transversalement en arrière des yeux et des auricules, et au niveau de l'orifice génital (fig. 1A). Le tronçon médian ainsi obtenu va édifier

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un blastème céphalique vers l'avant, et un blastème caudal vers l'arrière (fig. 1B). Nous avons aussi utilisé les blastèmes de queue obtenus à partir de la tête (fig. 1C).

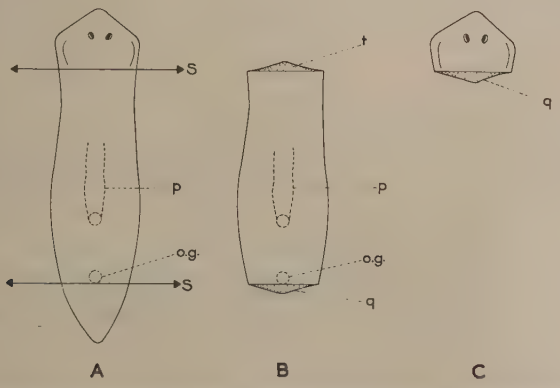

FIG. 1. Sections et formation des blastèmes. A. Sections pratiquées sur une planaire. B. Régénération du tronçon médian. C. Régénération de la tête. *o.g.*, orifice génital ♂; *p*, pharynx; *q*, blastème caudal; *s*, niveaux des sections; *t*, blastème céphalique.

Les blastèmes sont prélevés deux à trois jours après l'opération. A ce stade de la régénération, ils se présentent sous forme d'une petite languette étroite, non pigmentée et translucide. Aucune différenciation n'est encore visible.

Milieu de culture

La solution physiologique utilisée est le liquide de Holtfreter. Nous avons essayé des concentrations variées de cette solution (1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{10}$). Les plus favorables à la cicatrisation des explants sont les concentrations 1 et $\frac{1}{2}$.

Les bourgeons sont placés sur un milieu de culture solide et nutritif, contenant: 5 parties de gélose à 1 pour cent dans la solution de Holtfreter; 4 parties de solution de Holtfreter additionnée de glucose à 1 pour mille; 3 parties d'extrait d'embryon de poulet de 9 jours, dilué à 50 pour cent dans la solution de Holtfreter. L'extrait d'embryon s'est montré favorable à la survie des bourgeons. Dans certains cas, nous avons enrichi le milieu en y ajoutant une solution de plusieurs acides aminés.

Toutes les cultures ont été faites à une température de 18–20° C.

Asepsie des cultures

L'épiderme des bourgeons est couvert de germes qui risquent d'infecter les milieux et qu'il faut éliminer. Comme l'a fait Murray (1927, 1931), nous exposons les planaires entières, avant de prélever les bourgeons, aux rayons ultra-violets. Pendant le traitement, qui dure 4–5 minutes, les planaires sont recouvertes d'un film d'eau aussi mince que possible. Ensuite les animaux sont rincés assez longuement (30 minutes environ) dans 2 bains successifs d'eau stérile additionnée

de pénicilline. Alors seulement les bourgeons sont prélevés et placés sur le milieu qui contient également de la pénicilline.

Toutes ces précautions nous ont permis d'avoir des cultures stériles. D'autre part, ni les rayons ultra-violets, ni l'antibiotique utilisé, ne sont nuisibles aux planaires, dont le pouvoir de régénération, en particulier, reste intact.

RÉSULTATS

Nous avons fait des expériences de deux types: (1) culture de bourgeons de tête ou de queue isolés; et (2) culture d'associations de bourgeons de tête ou de queue.

CULTURE DE BLASTÈMES ISOLÉS

Au moment du prélèvement, le blastème présente une très grande surface de section. Parfois, les cellules du blastème commencent à s'échapper par la blessure et se dispersent à la surface du milieu. Mais après 24 heures de culture, la blessure est parfaitement cicatrisée, et les tissus cessent de migrer.

Les jours suivants, le blastème en culture devient un petit nodule plus ou moins sphérique, qui s'aplatit ensuite et prend la forme d'une lentille bien ronde.

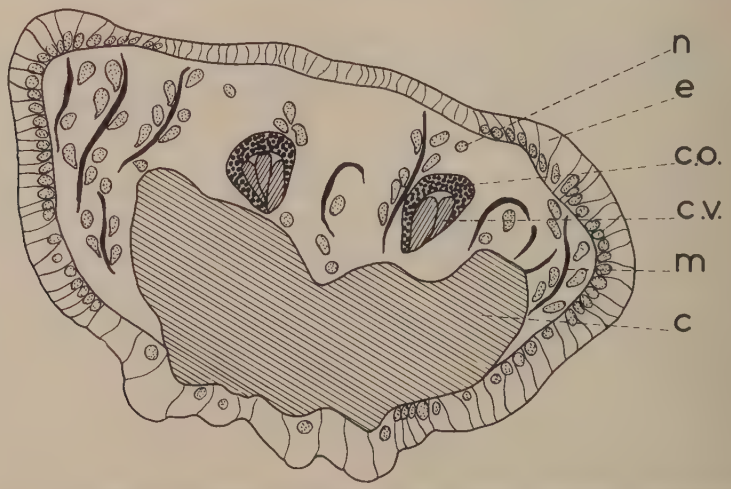

FIG. 2. Coupe d'un blastème céphalique ayant différencié un cerveau et 2 yeux. *c*, cerveau; *c.o.*, cupule oculaire; *c.v.*, cônes visuels; *e*, épiderme; *m*, muscles; *n*, néoblastes.

Dans cet état, les blastèmes survivent en moyenne 10 à 15 jours. Toutefois, certaines cultures ont pu être prolongées jusqu'à 3 ou 4 semaines. Pendant tout ce temps, les explants se sont maintenus à leur taille initiale. Nous n'avons pas observé de croissance notable. Par contre, les blastèmes sont capables de se différencier en culture. Les principales différenciations que nous avons remarquées sont les suivantes:

(*a*) Après 3 jours de culture, les explants commencent à se pigmenter. Le pigment apparaît sous forme de grains épars qui deviennent de plus en plus nombreux. La pigmentation progressive des bourgeons de régénération en culture est très semblable à celle que l'on observe *in vivo*. Toutefois, même après 4 semaines de culture, jamais les explants n'ont atteint une coloration aussi intense que celle de tissus adultes.

(*b*) Le 4e ou le 5e jour après la mise en culture, les bourgeons commencent à bouger. On observe des contractions particulièrement nettes lorsqu'on les éclaire fortement. Des muscles se sont donc différenciés. Leur existence est confirmée par l'observation au microscope (fig. 2).

(*c*) Le troisième type de différenciation ne concerne que les blastèmes antérieurs (les têtes présomptives). Sept à huit jours après le début de la culture, on voit apparaître une ou deux taches pigmentaires, nettement plus importantes

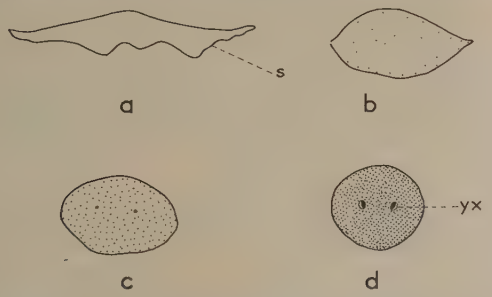

FIG. 3. Différenciation *in vitro* d'un blastème céphalique. *a*—mise en culture; *b*—48–72 heures; *c*—8 jours; *d*—10–15 jours. *s*, surface de section; *yx*, yeux.

que les grains de pigment qui colorent l'ensemble de l'explant. Les jours suivants, ces taches deviennent des yeux bien différenciés (fig. 3). Dans l'ensemble, la différenciation des yeux en culture se fait entre le 8e et le 15e jour, alors qu'*in vivo*, les yeux apparaissent dans un blastème céphalique environ 6 jours après le début de la régénération.

TABLEAU 1

Culture de blastèmes céphaliques isolés

	Nombre total de blastèmes mis en culture	*Blastèmes non cicatrisés (morts avant le 4e jour de culture)*	*Blastèmes cicatrisés normalement (après 24 heures de culture)*	*Blastèmes ayant différencié des yeux en culture (entre le 8e et le 15e jour)*
Série I milieu standard	42	8	34	22
Série II milieu avec acides aminés	26	2	24	13

Le tableau 1 donne, à titre d'exemple, les résultats de deux séries expérimentales: la série I sur le milieu standard (jus d'embryon), et la série II sur un milieu enrichi en acides aminés (jus d'embryon, et une solution de 9 acides aminés).

Lorsqu'on cultive des blastèmes céphaliques pris à des niveaux situés plus caudalement (en arrière du pharynx, par exemple), la proportion des yeux différenciés en culture est nettement plus faible que dans le cas des blastèmes formés à un niveau très antérieur. Sur 18 bourgeons pris au niveau de la bouche, 2 seulement ont différencié des yeux, après 15 jours de culture. Seize bourgeons pris au niveau de l'orifice génital n'ont donné aucune différenciation oculaire. Comme l'a montré Brønsted (1956), il existe un gradient le long du corps de la planaire, le 'time-graded regeneration-field': plus on s'éloigne de la tête, plus la régénération des yeux se fait tard. Ceci explique pourquoi, en culture, les blastèmes pris à des niveaux postérieurs à la bouche n'ont pas le temps de différencier des yeux avant qu'ils ne commencent à se désagréger.

L'examen histologique a montré que tous les blastèmes céphaliques qui ont différencié des yeux en culture, et qui ont été coupés et colorés, possèdent un cerveau (fig. 2). Ce résultat est en accord avec ceux de Lender (1950). Selon cet auteur, en effet, le cerveau des planaires émet une substance nécessaire à la différenciation des yeux.

Dans les bourgeons postérieurs (les queues présomptives) que nous avons cultivés, nous n'avons jamais obtenu aucune différenciation oculaire ni nerveuse. Il semble donc que, dans les conditions de nos expériences, seuls les blastèmes céphaliques soient capables de donner un cerveau et des yeux.

Nous avons vu que la culture de blastèmes de régénération isolés donne 3 types de différenciation: pigment, muscles, yeux et cerveau dans les bourgeons antérieurs. Des expériences de culture de plusieurs blastèmes associés ont apporté de nouveaux résultats. Un quatrième type de différenciation a été obtenu, en effet, à partir de certaines associations.

CULTURE DE BLASTÈMES ASSOCIÉS

Nous avons utilisé des blastèmes céphaliques et caudaux du même âge (2 à 3 jours) que dans les expériences précédentes. Deux ou plusieurs bourgeons, placés sur le milieu de culture, sont ensuite accolés par leurs surfaces de sections. Nous cultivons ces associations exactement de la même manière que les bourgeons isolés. Les divers éléments fusionnent rapidement et avec une grande facilité: après 24 à 48 heures, l'ensemble est en général parfaitement cicatrisé. Si, au départ, les surfaces de sections des différents bourgeons coïncident assez exactement, la cicatrisation est même plus aisée que dans le cas des bourgeons isolés. La survie est également plus longue en général, lorsque les blastèmes sont cultivés en associations: elle est de 20 à 25 jours en moyenne, si les blastèmes ont fusionné intimement.

Nous avons réalisé les associations suivantes: 2, 3 ou 4 blastèmes céphaliques; 2, 3 ou 4 blastèmes caudaux; 1 blastème céphalique avec 1 ou 2 blastèmes caudaux.

Associations de blastèmes céphaliques

L'ensemble prend une forme régulièrement arrondie, et des yeux se différencient dans le délai habituel. Il y a 1 ou 2 yeux par blastème. On obtient donc une sorte de tête ronde, aplatie, avec des yeux en surnombre (fig. 4).

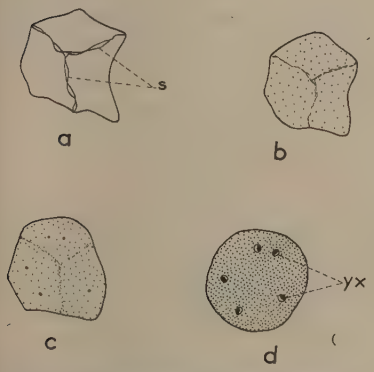

FIG. 4. Différenciation *in vitro* d'une association de 3 blastèmes céphaliques. Même légende que fig. 3.

Associations de blastèmes caudaux

L'évolution de la forme est la même, mais il n'y a jamais d'yeux.

Associations de blastèmes céphaliques et caudaux

Ce type d'association présente un changement de forme très caractéristique pendant les 8 à 10 premiers jours de la culture. En effet, quelle que soit sa forme au départ, un tel ensemble a toujours une forte tendance à s'allonger dans le sens céphalo-caudal. Les contours, d'abord irréguliers, s'arrondissent après le 3e jour, et, en s'allongeant, l'association finit par acquérir la forme d'une très petite planaire. La partie céphalique présomptive de l'ensemble constitue la tête, le ou les blastèmes caudaux la queue (fig. 5).

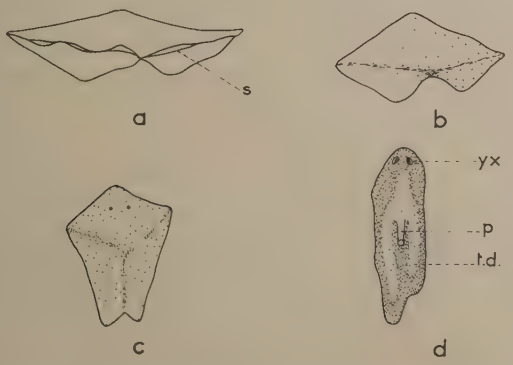

FIG. 5. Différenciation *in vitro* d'une association d'1 blastème céphalique avec 2 blastèmes caudaux. Même légende que fig. 1 et 3; *t.d.*, tube digestif.

Les différenciations déjà observées dans les expériences sur les blastèmes cultivés seuls, apparaissent dans les délais habituels: pigment, muscles, yeux dans le blastème céphalique. Nous avons obtenu un 4e type de différenciation dans ces associations de blastèmes céphaliques et caudaux. Entre le 10e et le 15e jour de culture, un pharynx apparaît dans la partie médiane de ces petites planaires. Se raccordant au pharynx, une ébauche de tube digestif à 3 branches a pu être vue par transparence dans certains cas. Sur 32 associations, 10 se sont ainsi différenciées en planaires pourvues d'un pharynx. Il faut noter qu'en culture le pharynx est apparu 3 à 4 jours après la formation des yeux, alors que, *in vivo*, les yeux et le pharynx se différencient presque simultanément dans un blastème de régénération.

TABLEAU 2

Culture d'associations de blastèmes céphaliques et de blastèmes caudaux

Nombre total d'associations mises en culture	*Associations non réussies (blastèmes non fusionnés, ou non cicatrisés)*	*Associations réussies (blastèmes fusionnés après 24 heures de culture)*	*Associations ayant survécu au delà du 10e jour de culture*	*Associations ayant différencié des yeux*	*Associations ayant différencié un pharynx*
34	2	32	14	14	10

Ces petites planaires nées *in vitro* semblent très bien adaptées au milieu de culture, où elles survivent 8 à 10 jours. D'autres ont été transplantées dans un milieu liquide (solution de Holtfreter diluée): elles s'y déplacent activement, mais n'y survivent pas plus de 4 à 5 jours. Le tableau 2 donne les résultats de la série expérimentale ayant différencié des pharynx.

CONCLUSION

L'ensemble des résultats obtenus au cours de ces recherches, démontre que la culture *in vitro* des blastèmes de régénération de planaires est possible: s'ils sont placés dans un milieu approprié, les bourgeons sont capables de survivre séparés de l'organisme qui leur a donné naissance. La méthode de culture utilisée permet également leur différenciation *in vitro*.

Une conclusion se dégage nettement des résultats de ces expériences: les blastèmes de régénération, même très jeunes, sont déjà *déterminés*, et leur polarité est fixée. En effet, un bourgeon antérieur donne toujours une tête, un bourgeon postérieur jamais. Pour la différenciation d'un pharynx et d'un tube digestif, la réunion d'un bourgeon céphalique et d'un bourgeon caudal est nécessaire. Pourquoi un blastème céphalique seul n'est-il pas capable de donner un pharynx? Il a été prouvé, en effet, qu'une tête induit normalement la différenciation d'un pharynx, et l'on peut se demander pourquoi il n'en est pas ainsi en culture. On doit admettre qu'un seul bourgeon n'apporte pas assez de matériel cellulaire pour permettre la différenciation d'organes digestifs, en plus des

différenciations nerveuse et oculaire. Pourtant, les associations de plusieurs blastèmes céphaliques sont aussi volumineuses que les associations mixtes d'un blastème céphalique et d'un blastème caudal, et ne peuvent cependant pas différencier un pharynx. Sans doute une telle association de 3 ou 4 blastèmes céphaliques, une fois différenciée, pourvue de ses cerveaux et de ses yeux, est-elle comparable à ces formations hétéromorphiques que l'on obtient parfois *in vivo*, les têtes bipolaires, par exemple. Dans ces cas spéciaux non plus, la reconstitution d'un pharynx n'est en général plus possible. L'association d'un blastème céphalique avec un blastème caudal, au contraire, tout en fournissant un matériel cellulaire suffisamment abondant, donne à l'ensemble les moyens d'accomplir un développement harmonieux qui conduit à l'édification d'une petite planaire pourvue de ses principaux organes.

RÉSUMÉ

1. De jeunes blastèmes de régénération de planaires ont été cultivés *in vitro* à l'aide de la méthode de culture de Wolff & K. Haffen, légèrement modifiée.

2. *Culture de blastèmes isolés.* Les blastèmes en culture se cicatrisent et survivent sur le milieu pendant 10 à 15 jours. Le milieu utilisé ne permet pas la croissance, mais les différenciations suivantes ont été obtenues: pigment (3^{e} jour); muscles (4^{e}–5^{e} jours); et yeux et cerveau (8^{e}–15^{e} jours). Ces 3 types de différenciation ont été observés dans les blastèmes de régénération antérieure (futures têtes), les 2 premiers seulement dans les blastèmes postérieurs (futures queues).

3. *Culture de blastèmes associés.* Une association de 2 ou plusieurs blastèmes céphaliques donne une tête avec des yeux en surnombre. Une association de 2 ou plusieurs blastèmes caudaux ne différencie jamais d'yeux ni de cerveau. Une association d'l blastème céphalique avec 1 ou 2 blastèmes caudaux donne une petite planaire pourvue d'un pharynx et d'un tube digestif.

4. Les résultats des expériences démontrent d'une part que la culture *in vitro* de jeunes blastèmes de régénération de planaires est possible, d'autre part que les blastèmes sont déterminés très précocement: les blastèmes antérieurs donnent des têtes, les blastèmes postérieurs au contraire ne sont pas capables de donner des différenciations céphaliques. Les blastèmes antérieurs associés aux blastèmes postérieurs reconstituent de petites planaires complètes.

SUMMARY

1. Young regeneration blastemata of Planarians have been cultivated *in vitro* on an agar-embryo extract culture medium which is a modification of the organ culture medium devised by Wolff & Haffen.

2. Transverse cuts were made in whole worms and 3 days later the blastemata were isolated on the culture medium, where they survive for 10–15 days. There is no visible growth, but the observations reveal pigment formation by the third day, the appearance of muscles on the fourth or fifth day, and the presence of

the eyes and brain by the tenth day. All three types of differentiation have been observed in the cultures of head blastemata, but only pigment and muscles are present in the cultures of tail blastemata.

3. When two or more head blastemata are cultured together they differentiate into a larger head with supplementary eyes. The association of two or more tail blastemata does not result in the differentiation of eyes or brain. The combination of one head blastema with one or two tail blastemata in such cultures gives rise to a small planarian with a pharynx and a gut.

4. The method of culturing regeneration blastemata *in vitro* reveals that the fate of the blastemata is determined quite early. A head blastema can differentiate cephalic structures, whereas a tail blastema cannot. However, if a head blastema is associated with a tail blastema in culture, then they differentiate into a complete planarian.

TRAVAUX CITÉS

BRØNSTED, H. V. (1956). Experiments on the time-graded regeneration-field in Planarians. *Biol. Medd. Abb.* **23**, 1–39.

DUBOIS, F. (1949). Contribution à l'étude de la migration des cellules de régénération chez les planaires dulcicoles. *Bull. biol.* **83**, 213–83.

LENDER, TH. (1950). Démonstration du rôle organisateur du cerveau dans la régénération des yeux de la Planaire *Polycelis nigra* par la méthode des greffes. *C.R. Soc. Biol. Paris*, **144**, 1407–9.

MURRAY, M. (1927). Cultivation of planarian tissues *in vitro*. *J. exp. Zool.* **47**, 467.

—— (1931). *In vitro* studies of planarian parenchyma. *Arch. exp. Zellforsch.* **11**, 656–68.

WOLFF, ÉT., & DUBOIS, F. (1947). Sur une méthode d'irradiation localisée permettant de mettre en évidence la migration des cellules de régénération chez les Planaires. *C.R. Soc. Biol. Paris*, **141**, 17–18.

—— & HAFFEN, K. (1952). Sur une méthode de culture d'organes embryonnaires *in vitro*. *Tex. Rep. Biol. Med.* **10**, 463–72.

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L'action inductrice du chordo-mésoblaste au cours de la gastrulation et de la neurulation et les effets de la culture *in vitro* sur ses manifestations[1]

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AVEC UNE PLANCHE

INTRODUCTION

La voûte archentérique contient deux zones distinctes aussi bien du point de vue de leur évolution que de l'action inductrice qu'elles exercent sur l'ectoblaste sus-jacent (Lehmann, 1938, 1941, 1945; Dalcq, 1946, 1947; Gallera, 1949; et d'autres). La zone antérieure, ou plus exactement la plaque préchordale, induit, éventuellement avec le concours de l'entoblaste pharyngien (Holtfreter, 1938; Hoessels, 1957), le cerveau antérieur (acrencéphale). L'induction du cerveau postérieur (deutencéphale) et de la moelle épinière revient à la chorde et au mésoblaste adjacent.

Cependant les recherches expérimentales de Nieuwkoop (1952) et de ses collaborateurs, notamment de Johnen (1956), ont démontré irréfutablement que l'agent responsable de l'induction des structures acrencéphaliques est répandu tout le long de la voûte archentérique. Dans son interprétation d'ensemble Nieuwkoop décompose le phénomène en deux phases, phase de l'activation et phase de transformation. Dans la première la formation d'un champ-gradient prosencéphalique serait déclenchée dans l'ectoblaste, lequel serait, par la suite, capable d'autodifférenciation aboutissant à la constitution du cerveau antérieur. Le processus de l'activation impliquerait donc la loi du 'tout ou rien'. La transformation des prédispositions acrencéphalogènes en tendances à former du cerveau postérieur et de la moelle serait due à une action beaucoup plus tardive et propre à la chorde dorsale.

Les résultats expérimentaux obtenus par Johnen semblent de prime abord confirmer brillamment cette interprétation. Les expériences de cet auteur s'appliquent à deux espèces d'Urodèles, *Amblystoma mexicanum* et *Triturus vulgaris*. Des fragments de la voûte archentérique excisés de la région deutoméritique (rhombencéphalique) de jeunes neurulas sont introduits entre deux lambeaux

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d'ectoblaste prélevé sur de très jeunes gastrulas. Ces 'sandwiches' sont cultivés en solution de Holtfreter pendant des temps variables, ensuite ouverts pour retirer l'inducteur. Cet ectoblaste, soustrait à l'action inductrice plus ou moins précocement, est alors élevé en solution de Holtfreter jusqu'à ce que les structures neurales induites soient convenablement différenciées.

Dans ces conditions une très courte induction (5 minutes au minimum) a déjà déclenché quelquefois la formation de structures neurales dans l'ectoblaste de l'Axolotl. Chez le Triton, dont l'ectoblaste réagit plus lentement, un contact durant quatre heures au moins s'est révélé indispensable. Le résultat le plus important est que les prestations de l'ectoblaste soumis à une action inductrice à peine supra-liminaire ont été de nature exclusivement acrencéphalique, tandis que l'induction beaucoup plus longue se solde toujours par la formation du cerveau antérieur et postérieur à la fois. Dans les expériences de contrôle, où l'inducteur a été laissé en permanence dans les 'sandwiches', l'ectoblaste en contact avec le chordo-mésoblaste a toujours fourni du rhombencéphale et du cerveau antérieur construit aux dépens des parties plus périphériques de l'explantat.

Ces rapports spatiaux entre l'inducteur et les structures neurales confirment la thèse de Nieuwkoop (1952), selon laquelle l'agent inducteur acrencéphalogène se répand dans l'ectoblaste plus largement que celui responsable de la formation du cerveau postérieur et de la moelle.

Quant au fait que les prestations de l'ectoblaste soumis à une induction de courte durée n'ont été que de nature acrencéphalique, Johnen y voit une confirmation de la thèse de Nieuwkoop. Dans ces cas, seulement la première phase de l'induction, phase de l'activation, aurait eu le temps de se réaliser. A mon avis, pourtant, une autre interprétation de ce résultat pourrait convenir. Nieu & Twitty (1953) ont dûment démontré que les fragments de la voûte archentérique cultivés *in vitro* diffusent des substances inductrices actives. L'analyse de divers hétéro-inducteurs (extraits de tissus adultes) a amené Toivonen (1950) et Kuusi (1951) à la conclusion que l'induction du cerveau antérieur est due à des substances chimiquement plus simples, plus diffusibles et au poids moléculaire moindre que celles responsables de la formation du cerveau postérieur et de la moelle. Quoiqu'en principe l'application directe des conclusions de cette analyse au processus de l'induction normale puisse paraître hasardeuse, je ne crois pas qu'une telle réserve se justifie dans le cas présent. En effet, les expériences de Nieuwkoop et de Johnen, expériences faites avec le chordo-mésoblaste vivant, aboutissent à une conclusion en accord parfait avec cette hypothèse. S'il en est ainsi, les substances inductrices acrencéphalogènes agissent probablement sur l'ectoblaste plus rapidement que les secondes. L'interruption du flux inducteur au moment opportun devrait permettre de dissocier artificiellement les effets de l'action inductrice acrencéphalogène de ceux provoqués par le pouvoir inducteur deutencéphalogène dont le chordo-mésoblaste serait doté d'emblée. Évidemment, cette dissociation ne serait possible que dans les cas où la puissance acren-

céphalogène du chordo-mésoblaste est suffisante. D'autre part, elle serait d'autant plus facile à réaliser que l'ectoblaste réagit plus promptement aux stimuli inducteurs. Or, Damas (1947) et Denis (1957), en soustrayant plus ou moins précocement l'ectoblaste à l'action inductrice d'une région beaucoup plus caudale de la voûte archentérique que celle utilisée dans les expériences de Johnen, n'ont jamais observé la formation de l'acrencéphale. Soulignons encore que, dans les expériences de ce dernier auteur, l'induction de l'acrencéphale seul s'est révélée plus facile à réaliser chez l'Axolotl que chez le Triton dont le développement se caractérise par une lenteur relative de l'induction.

Quoi qu'il en soit, il faut reconnaître que la formation de l'acrencéphale, obtenue parfois à la suite d'un contact extrêmement court avec le chordo-mésoblaste, semble plaider en faveur de la thèse de Nieuwkoop selon laquelle l'induction du cerveau antérieur ne serait en réalité qu'une simple activation des tendances évolutives latentes de l'ectoblaste. Malheureusement, cet argument est à mon avis d'autant plus sujet à caution que les expériences de Johnen ont été faites dans des conditions manifestement extra-normales. D'une part, l'ectoblaste très jeune a été soumis à l'action du chordo-mésoblaste d'âge avancé et, d'autre part, le processus d'induction s'est opéré *in vitro*. Ainsi deux facteurs nouveaux ont été introduits sans que leur rôle éventuel ait été pris en considération. Or, les résultats de nombreuses études expérimentales semblent attester leur incontestable importance dans le cadre présent.

Comme nous le savons, le matériel préchordal présomptif prélevé sur une jeune gastrula et cultivé soit en 'sandwiches' ectoblastiques, soit dans le blastocoele d'autres embryons, se différencie en chorde et en muscles avec induction de structures neurales deutencéphaliques et spinales. La préchorde n'acquiert ses propriétés évolutives et inductrices caractéristiques qu'après son invagination (Okada & Takaya, 1942; Okada & Hama, 1943, 1945; Takaya, 1953; Hoessels, 1957). Quoique le chordo-mésoblaste ne manifeste pas une inversion si radicale de son pouvoir inducteur, j'ai pu constater dans un travail récent (1959) que sa capacité à induire le cerveau antérieur n'apparaît que vers la fin de la gastrulation. Cette aptitude est d'ailleurs toujours faible et dominée par le pouvoir inducteur deutencéphalogène et spinal propre à ce matériel. Dans la première série de ces expériences le neurectoblaste rhombencéphalique présomptif a été prélevé aux stades successifs de la gastrulation et de la neurulation et transplanté sur la face ventrale de jeunes neurulas. Mes greffons les plus jeunes étaient encore incapables de former du cerveau, les transplantats un peu plus âgés ont fourni du rhombencéphale, la formation de l'acrencéphale ne fut jamais observée. Dans la deuxième série d'expériences, des fragments d'ectoblaste ventro-animal prélevés au début de la gastrulation ont été implantés sur la région deutoméritique de la voûte archentérique de jeunes neurulas où ils ont été laissés durant des temps variables pour être ensuite transplantés sur la face ventrale d'autres neurulas. Quelques-uns des greffons soumis à l'induction relativement courte ont fourni du cerveau antérieur et des organes des sens lui

correspondant, les greffons laissés plus longtemps sur le substratum inducteur ont évolué en rhombencéphale. Cette série de mes expériences, où l'ectoblaste jeune a été mis en contact avec l'inducteur déjà évolué, a donné, contrairement aux expériences de la première série, des résultats comparables à ceux obtenus par Johnen. Il s'avère donc que l'apparition fréquente des formations neurales exclusivement acrencéphaliques dans des 'sandwiches' de cet auteur est au moins partiellement due à l'âge avancé du chordo-mésoblaste employé dans ses expériences.

Signalons encore que le pourcentage des prestations acrencéphaliques a été considérablement plus élevé dans les expériences de Johnen que dans les miennes. On peut se demander si la différence entre nos résultats peut être imputable à l'influence exercé par la culture *in vitro* soit sur le chordo-mésoblaste, soit sur l'ectoblaste, ou, enfin, sur ces deux formations. En effet, de nombreuses raisons le font croire. Le développement du chordo-mésoblaste enfermé dans des 'sandwiches' ectoblastiques est parfois anormal; ter Horst (1948) a même observé dans de telles conditions la transformation du matériel chordal présomptif en tissu nerveux. L'exposition de la face interne de l'ectoblaste prélevé sur les jeunes gastrulas de l'*Amblystoma punctatum* à l'action de la solution saline suffit déjà pour provoquer la formation des structures neurales acrencéphaliques dans cet ectoblaste (Barth, 1941). Chez d'autres espèces d'Urodèles dont l'ectoblaste est moins sensible le même résultat a pu être obtenu en utilisant des solutions salines à tendances légèrement cytolytiques (Holtfreter, 1944, 1947; Karasaki, 1957). Une cytolyse très discrète provoquerait la libération des substances inductrices au sein même de cellules ectoblastiques et aux dépens de certains de ses composants plasmatiques. Il n'est donc pas exclu que la culture *in vitro* ait sur l'ectoblaste une faible influence tendant à diriger son développement vers la formation de l'acrencéphale. Cette action du milieu externe serait sous-liminaire; cependant, additionnée à l'impulsion inductrice due à un court contact avec le chordo-mésoblaste, elle pourrait devenir plus ou moins efficace.

Afin de répondre à ces questions, j'ai repris mes recherches sur l'évolution des capacités inductrices du chordo-mésoblaste, mais, cette fois-ci, ce phénomène est étudié hors de l'organisme, à l'aide de la méthode de 'sandwiches' ectoblastiques.

Ce n'est pas tellement les résultats mêmes de ces expériences qui comptent, que leur confrontation avec ceux obtenus sur les embryons entiers et ceux de Johnen, laquelle démontrerait l'influence exercée par la culture *in vitro* sur le processus d'induction.

Rappelons d'autre part que la face interne de la voûte archentérique, correspondant à la face externe de la zone marginale d'une jeune gastrula, est probablement revêtue par le 'coat' (Holtfreter, 1943, 1944). Quoique ce dernier n'ait pas pu être décelé morphologiquement, même à l'aide du microscope électronique (Karasaki, 1959), sa grande importance physiologique ne peut être mise en question. C'est pourquoi je me suis également efforcé dans le présent travail de

savoir si les deux faces (externe et interne) de cette voûte exercent une action inductrice identique.

MATÉRIEL ET TECHNIQUE

Une seule espèce d'Urodèles, *Triturus alpestris*, a servi aux expériences. Les opérations sont effectuées aseptiquement sur fond d'agar dans la solution de Holtfreter avec adjonction d'un sulfamide (Cibazol) dans la proportion de 0,5 pour mille. Ce produit ne modifie pas le pH de la solution.

Les donneurs sont immergés préalablement dans une solution de bleu de Nil très diluée. Ce traitement, étant appliqué aux blastulas avancées, le matériel embryonnaire invaginé se trouvera plus tard coloré. Des rectangles, contenant du chordo-mésoblaste deuto- et cormoméritique (cérébral postérieur et troncal), sont excisés de la voûte archentérique de gastrulas et de jeunes neurulas. Les premières sont soit au stade de grand blastopore circulaire, soit de blastopore encore rond mais déjà nettement rétréci; les neurulas sont opérées au moment de l'apparition du liséré pigmenté délimitant la plaque neurale ou quand les bourrelets médullaires s'élèvent en relief. Le procédé opératoire consiste à mettre à jour (sur des donneurs colorés au bleu de Nil), à l'endroit choisi, la voûte archentérique, afin d'y prélever un rectangle de matériel inducteur et à l'envelopper entre deux lambeaux d'ectoblaste ventro-animal prélevé sur des gastrulas (au blastopore en croissant ou en fer à cheval). La fenêtre pratiquée dans le feuillet externe est comblée par un morceau d'ectoblaste provenant d'un autre embryon. Ces greffons, non colorés, sont toujours bien visibles sur les donneurs teintés en bleu. Sur les neurulas la localisation du matériel prélevé, d'ailleurs facile à ce stade, peut être contrôlée rétrospectivement grâce à l'observation de la position de ces greffons. Cette localisation s'avère plus difficile à déterminer sur des gastrulas. Les donneurs sont alors disséqués sous la loupe binoculaire le jour suivant, quand les bourrelets médullaires sont déjà formés. La zone d'excision se cicatrise lentement et permet encore à ce stade, où les relations topographiques sont plus nettes, de préciser la région de prélèvement du matériel inducteur. Seuls les explantats où cette localisation correspondait aux conditions exigées sont pris en considération. Toutefois, dans certains explantats le matériel deutoméritique prédomine; dans d'autres, plus rarement, le chordo-mésoblaste déjà troncal prévaut.

Les 'sandwiches' sont transportés dans la solution de Holtfreter fraîche, mais toujours additionnée de Cibazol, où ils sont laissés durant 16 à 24 heures. Comme je l'ai constaté dans mon travail précédent (1959), chez le Triton alpestre l'induction est très lente: 10 heures de contact avec le chordo-mésoblaste sont insuffisantes, à l'exception de quelques cas rares, pour obtenir une réaction de la part de l'ectoblaste. Une induction dépassant 22 heures est toujours suivie par la formation exclusive du cerveau postérieur. Or, le développement des explantats est un peu plus lent que celui des greffons (Gallera, 1947; Woellwarth, 1952; Nieuwkoop & Nigtevecht, 1954; Denis, 1957). J'ai donc

prolongé intentionnellement le temps d'induction dans les expériences présentes. La période d'induction achevée, les deux feuillets du 'sandwich' sont décollés pour ôter l'inducteur. Cette opération s'est révélée difficile; l'ectoblaste quelques heures après la préparation d'un 'sandwich' se plisse souvent irrégulièrement et dans quelques cas une petite portion de l'inducteur, coincée dans ces replis, n'a pas pu être rétirée. Ces expériences techniquement déficientes serviront à titre de contrôle.

Les deux lambeaux ectoblastiques, dorsal et ventral, sont couverts d'épiblaste prélevé sur la face ventrale de neurulas. Cette intervention a pour but de protéger la face interne de l'ectoblaste de l'action directe de la solution saline. J'ai utilisé de l'épiblaste déjà âgé et incapable de différenciation neurale pour éviter la complication inutile des résultats expérimentaux, car l'ectoblaste jeune aurait réagi à l'action inductrice exercée par les formations cérébrales induites dans le feuillet provenant du 'sandwich'.

Les deux explantats définitifs sont transportés séparément dans deux récipients contenant de la solution de Holtfreter additionnée de Cibazol. L'un de ces explantats, dit dorsal, contient donc l'ectoblaste qui était auparavant en contact avec la face du chordo-mésoblaste tournée normalement vers le feuillet externe; l'autre, ventral, est formé d'ectoblaste contre lequel a été appliquée la face inductrice formant le plafond de la cavité archentérique.

Les explantats sont élevés de 11 à 17 jours à la température de 15–20° C., ensuite fixés au Bouin et examinés sur les coupes sériées de 8 μ (coloration à l'hémalun et le mélange d'éosine et d'orange). Dans les cas où les structures neurales sont très irrégulières on a établi des reconstructions graphiques.

RÉSULTATS EXPÉRIMENTAUX

L'ectoblaste préalablement soumis à une induction plus ou moins courte est accolé à un fragment d'épiblaste ventral d'une neurula. Rapidement les bords des deux morceaux se soudent. Par l'absorption de liquide ambiant et la sécrétion active des cellules ectoblastiques, les explantats se dilatent en ballonets qui éclatent souvent en cours d'élevage et s'aplatissent passagèrement pour se regonfler après cicatrisation. En fin d'élevage, leur forme est irrégulière, leur surface chagrinée est munie parfois d'appendices évoquant des balanciers ou plus rarement des nageoires, présentes exclusivement sur les explantats dont l'ectoblaste fut mis en contact avec l'inducteur jeune. Quelques-uns de ces derniers explantats subissent un allongement considérable, phénomène jamais observé sur les explantats constitués d'ectoblaste soumis à l'action d'un inducteur âgé, prélevé sur des neurulas. Au bout du cinquième ou septième jour, la plupart des explantats fournissent des cellules pigmentaires et souvent des structures neurales et sensorielles transparaissent. A ce stade, beaucoup d'explantats (la moitié environ) commencent à dégénérer et se désintègrent.

L'examen histologique du matériel survivant nous permet d'emblée de grouper les résultats en trois catégories.

Seize explantats présentent du matériel inducteur résiduel. Ces expériences déficientes, utilisées à titre de contrôle, seront décrites plus loin.

Parmi les explantats dont l'ectoblaste fut mis en contact avec l'inducteur âgé, un dixième n'ont pas réagi à l'induction. Ils sont formés de l'épiblaste atypique épais, pluricellulaire, creusé de cavités, éventuellement remplies de détritus cellulaire et de mucus.

Dans toutes les autres expériences, on observe au moins la formation d'ecto-mésenchyme qui a permis une belle différenciation de l'épiblaste avec une éventuelle induction, même en absence de structures neurales, de cellules glandulaires. Si les structures neurales se sont formées, on distingue aisément une nette gradation dans l'état de leur développement. Les unes se présentent sous la forme de petites vésicules composées d'éléments cellulaires peu différenciés et surchargés de vitellus, les autres forment de grands complexes cérébraux et occasionnellement oculaires. De temps en temps un rhombencéphale bien reconnaissable se prolonge en un cordon d'aspect médullaire étroit présentant des segments oblitérés. Rarement enfin, une structure neurale rudimentaire, indéfinissable morphologiquement, accompagne un complexe cérébral bien constitué; toutefois ces formations sont toujours séparées l'une de l'autre. Les complexes cérébraux induisent souvent des cristallins ou 'cristallinoïdes' et des placodes olfactives, les otocystes exceptionnellement formés se limitant à des rudiments.

Dans mes expériences l'ectoblaste fut soumis à une action inductrice de 16 à 24 heures. Cependant cette variation n'explique que partiellement la grande différence constatée entre les prestations des divers explantats. En effet, quoique la plupart des complexes cérébraux et sensoriels se soient constitués dans l'ectoblaste laissé en contact avec l'inducteur durant vingt heures et plus, certains n'ont donné qu'un peu d'ecto-mésenchyme. La nature des prestations dépend donc encore d'autres facteurs dont l'un, disons-le d'avance, est l'âge de l'inducteur. D'autre part, la répartition des résultats en fonction de l'âge du chordo-mésoblaste présente quelques cas aberrants qui prouvent qu'un facteur incontrôlable, lié à la technique expérimentale, joue ici un grand rôle. En effet, dans les 'sandwiches' recelant un fragment de l'inducteur, l'intimité de l'adhésion aux parois du 'sandwich', qui par surcroît gonfle rapidement et change continuellement de forme, est nécessairement fortuite.

La forme des structures neurales, fréquemment des plus irrégulières, ne permet qu'exceptionnellement le diagnostic morphologique direct. Souvent pourtant, la présence des formations régionales spécifiques, telles la vésicule optique, la paraphyse ou l'épiphyse, les cellules de Mauthner, et l'induction des placodes olfactives ou des otocystes, fournissent des critériums suffisants. La formation, si fréquente dans mes explantats, des cellules épiblastiques glandulaires, cellules qui sur la larve normale sont réparties dans la région antérieure de la tête et ne dépassent jamais le niveau du cerveau moyen, ne nous offre, malheureusement, aucun renseignement sur la nature des structures neurales obtenues

expérimentalement. Holtfreter (1933) a observé que, dans les transplantats, ces cellules peuvent accompagner le rhombencéphale et même les amas de cellules ganglionnaires; dans quelques-uns de mes explantats dépourvus de toute formation neurale, j'ai constaté la présence de ces cellules, induites par l'ecto-mésenchyme seul.

Un peu plus qu'un tiers des explantats ont donné des formations neurales dont la nature échappe à toute définition organogénétique. Ce groupe englobe deux sortes de structures neurales: les vésicules en voie de désintégration secondaire provoquée par l'hydropie excessive, et les formations plutôt neuroïdales que neurales dues à une induction insuffisante.

Dans le premier cas on a affaire à des vésicules dilatées dont la paroi est réduite sur de grandes étendues à une seule couche de cellules suraplaties; dans d'autres endroits elle est encore épaisse, mais, la substance intercellulaire fondamentale (Freedmann, 1953; Hess, 1953, 1955; Bairati & Tipoli, 1954) étant partiellement dissoute, sa texture est fortement relâchée. Entre les cellules, dont la différenciation neuronale est d'ailleurs normale, apparaissent des lacunes et les cellules manifestent une certaine tendance à se disperser.

Les structures neuroïdales se présentent sous des aspects des plus variés. Certaines assument la forme de nodules vaguement ganglionnaires, d'autres, assimilables à des vésicules à paroi épaisse surchargée de vitellus, rappellent par leur disposition et l'état de leur différenciation les ébauches neurales tout au début de leur formation; parfois enfin, se constituent des amas de neurones bien différenciés, mais leurs cellules périphériques s'aplatissent par endroits, en formant une sorte de toïle choroïdienne, qui, par décollement, forme des kystes latéraux (Planche, fig. 1). Notons encore que dans une même structure le degré de développement réalisé peut varier considérablement selon les régions: une zone localisée formée de neurones bien différenciés se retrouve entourée d'éléments dont l'évolution commence à peine.

Le tableau ci-joint rapporte les résultats individuels de mes expériences. Dès le premier examen, on observe qu'une différence entre les durées d'induction (8 heures au maximum) n'agit pas appréciablement sur les prestations des explantats. Une induction relativement courte, très proche de sa longueur minima (16 heures), suffit déjà pour déclencher la formation de structures neurales bien organisées; dans un cas, par exemple, du rhombencéphale s'est constitué après 17 heures d'induction seulement. D'autre part, la durée maximum (24 heures) de contact avec le chordo-mésoblaste se révèle encore insuffisante pour que son pouvoir inducteur puisse se manifester entièrement. Souvent les structures neurales induites dans ces conditions ne correspondent pas au caractère régional de l'inducteur. En effet, deux explantats de ce groupe n'ont fourni que de l'acrencéphale. Ainsi le temps d'induction choisi répond parfaitement aux conditions expérimentales exigées.

Contrairement à mes prévisions, les actions inductrices des faces ventrales et dorsales du chordo-mésoblaste ne présentent aucune différence. En revanche,

TABLEAU 1. *Stade du donneur au moment du prélèvement de l'inducteur*

	Grand blastopore circulaire									*Blastopore circulaire déjà rétréci*														
	Temps de contact avec l'inducteur																							
Temps:	20.15	20.30	20.30	20.30	20.45	21.30	22	24	24	17	18	19.45	20	20.15	20.45	20.45	21.15	21.15	21.30	21.30	22	22.30	22.45	23.30	24
No. de protocole:	4	56	57	94	60	97	92	33	34	62	61	74	81	87	93	59	76	82	58	68	72	31	96	49	27
	d v	d v	v	d	d	d	d	d v	v	d	d v	v	d v	v	v	v	v	v	d	d v	d	v	d v	d	d v
Acrencéphale								×					+											+	+ ×
Pl. olfactive													+											2	2 2
Œil								×																+	2 ×
Cristallin								×																	+ 2
Rhombencéphale	+	+ ×	×	+	+			+		+		×		×		×				×				+	
Ganglions		+ ×	×					+						×						×				+	
Otocyste								+						×											
Moelle		? ?	?																						
Str. neurale indefinie			×			+	+		×		+ ×		×				×		+	×	+	×	×		
Ecto-mésenchyme	+ ×	+ ×	×	+	+	+	+	+ ×	×	+	+ ×	×	+ ×	×	×	×	×	×	+	+ ×	+	×	+ ×	+	+ ×
Cel. pigmentaires	+ ×	+ ×	×	+	+	+	+	+ ×	×	+	+ ×	×	+ ×	×	×	×	×	×	+	×	+	×	+ ×	+	+ ×
Cel. glandulaires		×	×	+	+	+	+	+ ×	×	+	×	×	+ ×	×	×	×		×	+	×		×	+ ×	+	+ ×

	Sillon médian — liséré pigmenté									*Bourrelet en relief*														
Temps:	16	18.30	20	21	21	21.15	21.15	21.15	21.45	22.45	19	19	19	19.45	20	20.15	20.15	21	21.15	21.15	21.30	21.45	22.15	22.30	23	23
No. de protocole:	10	63	14	41	46	39	40	86	69	95	5	7	9	53	8	65	85	43	77	90	70	89	79	28	29	30
	d	d	d	d v	d	d v	d v	d	d v	d v	d v	v	d v	d v	d	v	d v	v	d	d	d	d v	d	d		d v
Acrencéphale					+	×	+ ×		+	×					+		×		+						×	+
Pl. olfactive					+	×	2 ×		2	2					+		×		+						×	2
Œil					+	3			2								×		+							+
Cristallin						2			2										+							2
Rhombencéphale						+	+																			
Ganglions																										
Otocyste																										
Moelle																										
Str. neurale indéfinie		+	+	×		×				×			×	+		×	+					×	+	+		
Ecto-mésenchyme	+	+	+	+ ×	+	+ ×	+ ×	+	+ ×	+ ×	+ ×	×	+ ×	+ ×	+	×	+ ×	×	+ ×	+	+	+ ×	+	+	×	+ ×
Cel. pigmentaires		+	+	×	+	+ ×	+ ×		+	+ ×	+		+	+	+				+					+		
Cel. glandulaires	+	+	+	×	+	+ ×	+ ×		+	+ ×			+ ×	+	+	×	+ ×	×	+ ×	+		+ ×		+	×	+

LÉGENDE: + = prestation des explantats dorsaux. × = prestation des explantats ventraux.

la nature des prestations obtenues et la puissance inductrice du chordo-mésoblaste sont en large mesure tributaires de l'âge de l'inducteur. Le chordo-mésoblaste de jeunes gastrulas, même si son action a été à peine supra-liminaire, induit surtout du rhombencéphale ($\frac{7}{8}$ de toutes les structures neurales se prêtant au diagnostic morphologique), alors que le même matériel, prélevé sur une neurula au stade des bourrelets médullaires, provoque la formation ou bien d'acrencéphale (Planche, figs. 2, 3) ou bien de structures morphologiquement indéfinissables.

En revenant à la puissance inductrice de la voûte archentérique, notons qu'elle diminue déjà à partir des stades avancés de la gastrulation.

Ces phénomènes, masqués plus ou moins sur le tableau d'ensemble par les cas aberrants dûs au contact fortuitement moins étroit entre l'inducteur et l'ectoblaste, ressortent très nettement sur le graphique (fig. 1 dans le texte), où les nombres des différents types d'inductions en pour-cent sont rapportés en

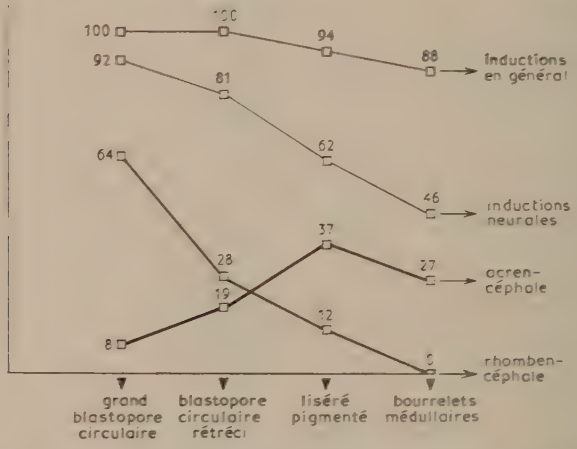


FIG. 1. Graphique des pourcentages d'induction de différents types en fonction de l'âge de l'inducteur.

ordonnée, et l'âge de l'inducteur au moment de son prélèvement en abscisse. Comme on le voit, les lignes correspondant respectivement au nombre de cas d'inductions neurales en général et à la fréquence des formations acrencéphaliques convergent rapidement jusqu'au début de la neurulation des donneurs de l'inducteur, et ensuite beaucoup plus lentement. Cependant ce dernier phénomène est probablement plus apparent que réel. L'action inductrice du chordo-mésoblaste de neurulas à bourrelets médullaires est déjà si faible que dans la moitié des cas environ elle ne suscite que la formation de structures neurales rudimentaires, sans caractère morphologique défini. Étant donné que toutes les autres formations neurales fournies par ce groupe d'explantats sont de nature acrencéphalique, il est légitime de supposer que la plupart des structures neurales

visiblement sous-induites ont également reçu une impulsion inductrice acrencéphalogène, quoique trop faible pour que le cerveau antérieur bien constitué puisse se développer.

Le développement des ébauches oculaires dans les complexes acrencéphaliques doit encore retenir notre attention. Les deux cupules optiques ne se sont formées que dans un cas exceptionnel; dans tous les autres l'ébauche oculaire est impaire. Le cerveau antérieur étant de taille réduite, l'ébauche oculaire reste souvent très petite. Cependant, parfois, nous assistons à unphénomène contraire: presque tout le matériel qui a réagi à l'impulsion inductrice se transforme en rétine. Celle-ci est alors très grande, irrégulièrement plissée et constituée presque exclusivement du feuillet sensoriel (Planche, fig. 4). Comme on le sait, la différenciation de la rétine est relativement précoce. La formation de complexes rétiniens beaucoup trop grands par rapport au cerveau est probablement l'effet d'une compétition physiologique au cours de laquelle l'ébauche à différenciation très spécifique, et précoce, s'est emparé de la plus grande part du matériel disponible.

Comme je l'ai déjà mentionné, 16 de mes expériences ont été techniquement défectueuses, une petite portion de l'inducteur étant restée attachée au feuillet 'dorsal' ou 'ventral' des 'sandwiches' ectoblastiques. Par hasard, dans une seule de ces expériences l'inducteur provenait d'une neurula, tandis que dans toutes les autres il a été prélevé sur des gastrulas. Le matériel inducteur laissé sur l'ectoblaste s'est différencié soit en petits îlots chordaux, soit en fibres musculaires, soit enfin en ces deux formations simultanément. Les parties de l'inducteur engagées dans les replis de l'ectoblaste étant les plus difficiles à retirer, les îlots chordaux se trouvent parfois au sein même des structures neurales formées par l'ectoblaste, dans la substance blanche de leurs parois.

Comme on pouvait le prévoir, tous ces explantats ont fourni des structures neurales mieux différenciées que celles formées par les explantats exclusivement ectoblastiques. Tous les explantats contenant une petite portion du chordo-mésoblaste prélevé sur des gastrulas ont fourni du rhombencéphale prolongé parfois par un court tronçon médullaire. En revanche, le seul explantat auquel est resté attaché un petit fragment de la voûte archentérique, provenant d'une neurula, n'a donné que du cerveau antérieur, muni de deux yeux et accompagné des deux placodes olfactives. Dans ce cas, le matériel inducteur a formé un tronçon chordal, court et coudé, et un petit amas de tissu musculaire. Comme on le voit sur la figure 4 de la Planche, ces formations adhèrent étroitement à l'une des ébauches oculaires. La formation du cerveau antérieur au voisinage immédiat de la chorde dorsale peut paraître de prime abord paradoxale; n'oublions pas pourtant que ce tronçon chordal est beaucoup plus petit que les cerveaux et les ébauches oculaires. Ces dernières formations ont été, certes, induites par le chordo-mésoblaste; mais la majeure partie de celui-ci a été enlevée précocement, avant qu'elle ait pu exercer pleinement son action inductrice, régionalement spécifique. Évidemment l'induction du cerveau antérieur par le chordo-mésoblaste

âgé implique la présence dans ce dernier de substances acrencéphalogènes. Comme je l'ai démontré dans mon travail précédent (1959), cette action acrencéphalogène du chordo-mésoblaste, quoique plus précoce, est dominée en fin de compte par le pouvoir inducteur deutencéphalique et médullaire de la région chordale de la voûte archentérique. Dans le cas présent, seule une très petite quantité de l'inducteur subsistant, son pouvoir n'a pas pu se manifester. Soulignons encore que dans les 15 cas où l'inducteur, maintenu en contact avec l'ectoblaste durant tout le temps de son élevage, fut plus jeune, prélevé sur des gastrulas, les explantats n'ont donné que du cerveau postérieur. Ainsi donc les résultats de ces expériences techniquement défectueuses étayent encore le fait essentiel démontré par mes expériences principales que le pouvoir inducteur du chordo-mésoblaste évolue et se modifie considérablement au moins à partir de la fin de la gastrulation.

DISCUSSION

J'avais entrepris, dans le précédent travail (1959) publié dans ce journal, l'étude des rapports entre le pouvoir inducteur du chordo-mésoblaste et son évolution dans le temps. Les expériences présentes prolongent et complètent cette étude. Dans le premier travail, j'ai recouru à la méthode des greffes; actuellement le pouvoir inducteur est analysé hors de l'embryon; la zone deutoméritique de la voûte archentérique est prélevée au cours de la gastrulation ou de la neurulation et combinée temporairement avec l'ectoblaste jeune. Les différences constatées entre les résultats de ces deux investigations révèlent l'importance des circonstances spéciales créées par chaque type d'expérimentation.

Avant d'aborder la discussion des problèmes que ces différences soulèvent, rappelons les faits essentiels qui se retrouvent concordants dans mes deux investigations, et qui corroborent certaines données déjà acquises, tout en apportant des précisions nouvelles.

Les expériences de Johnen (1956) — faites *in vitro* avec du chordo-mésoblaste prélevé sur des neurulas et de l'ectoblaste provenant des gastrulas — et les miennes, démontrent que l'acrencéphale ne peut se constituer dans l'ectoblaste à l'endroit même de son contact avec du chordo-mésoblaste que dans les cas où ce contact a été interrompu suffisamment tôt. Une action inductrice plus prolongée mène toujours à la formation du cerveau postérieur et, éventuellement, de la moelle.

D'autre part pourtant, mes expériences ont mis en évidence un fait nouveau et tout à fait incompatible avec l'interprétation de l'induction neurale proposée par Nieuwkoop (1952). Comme on s'en souvient, selon cet auteur, la première manifestation de l'action inductrice correspondrait à la formation d'un champ-gradient acrencéphalogène dans l'ectoblaste sus-jacent. Une action secondaire, tardive et prolongée, du chordo-mésoblaste serait indispensable pour convertir cette impulsion évolutive déclenchée dans l'ectoblaste en tendances à former du

rhombencéphale et de la moelle. Or mes expériences prouvent d'une façon irréfutable que ce pouvoir acrencéphalogène partiel n'appartient nullement d'emblée au chordo-mésoblaste; au contraire il ne devient manifeste que tardivement, au moment où chez l'embryon la puissance inductrice de la voûte archentérique est déjà nettement affaiblie et le processus même de l'induction achevé dans ses grandes lignes. Autrement dit, au moment où le matériel chordo-mésoblastique atteint, grâce aux mouvements d'invagination gastruléenne, la face interne de l'ectoblaste et commence à exercer son action sur lui, celle-ci est de nature deutencéphalogène et spinale. Comme le graphique sur la page 486 du travail présent le visualise clairement, le pouvoir inducteur du chordo-mésoblaste évolue et se modifie considérablement au cours de la gastrulation et de la neurulation. Il est intéressant de remarquer que cette évolution, quoique plus lente et restreinte, est de même nature que celle connue déjà depuis longtemps dans le matériel présomptif de la plaque préchordale; car même cette dernière est primitivement dépourvue de toute capacité d'induire du cerveau antérieur. Ainsi il s'avère que le pouvoir inducteur acrencéphalogène n'est jamais et nulle part primordial; au contraire il est toujours le résultat d'une évolution plus ou moins longue.

Dès les premiers essais d'analyse du processus d'induction hors de l'embryon, *in vitro*, nous savons que les deux faces de l'inducteur agissent sur l'ectoblaste. Jusqu'à présent pourtant, les capacités inductrices des deux faces de la voûte archentérique n'ont pas été, autant que je le sache, étudiées séparément et comparativement. Les expériences exposées dans ce travail comblent cette lacune: ces deux faces exercent (au moins s'il s'agit de la région deutoméritique) une action inductrice identique, aussi bien du point de vue qualitatif que quantitatif.

La comparaison des résultats de mes expériences précédentes et actuelles révèle que l'induction du cerveau antérieur par le chordo-mésoblaste s'avère plus facile à réaliser dans l'ectoblaste cultivé *in vitro* que dans les greffons implantés sur la voûte archentérique et transplantés ensuite sur la face ventrale d'un autre embryon. Dans le premier cas, non seulement la fréquence des prestations acrencéphaliques est manifestement plus élevée, mais on peut déjà les obtenir à partir du chordo-mésoblaste plus jeune. L'éventuelle supposition selon laquelle le mésoblaste ventral de l'hôte exercerait une faible influence 'caudalisante' sur les greffons soumis préalablement à une induction juxta-liminaire doit être écartée d'emblée. En 1947, j'ai exécuté de nombreuses transplantations des fragments de la plaque acrencéphalique soustraite précocement à l'action inductrice de son substratum. Aucun de ces greffons, implantés sur la face ventrale de jeunes neurulas, n'a fourni du rhombencéphale. Pourtant certains étaient de taille minime, et quelques-uns d'entre eux avaient subi une impulsion acrencéphalogène à peine supra-liminaire. En revanche, à de multiples occasions, l'action faiblement acrencéphalogène de la solution saline de Holtfreter s'est confirmée. Cette action, se ramenant probablement à la libération de substances inductrices au sein même des cellules ectoblastiques, et aux dépens

de certains de leurs composants plasmatiques, n'a pu être démontrée directement que chez une seule espèce d'Urodèles, à savoir *Amblystoma punctatum* (Barth, 1941). Chez les autres Urodèles, l'ectoblaste est moins sensible; cependant Nieuwkoop & Nigtevecht (1954), Hori & Nieuwkoop (1955) et Sala (1956) ont observé la formation de petits complexes acrencéphaliques aux bords de leurs 'sandwiches' préparés à partir d'ectoblaste et de chordo-mésoblaste de l'Axolotl. Or la partie périphérique d'un explantat est la plus exposée à l'action de la solution saline. Rapportons encore que ces complexes acrencéphaliques étaient tout à fait indépendants des structures neurales induites par le chordo-mésoblaste.

Indépendamment de cette action renforçant légèrement les tendances acrencéphaliques des structures neurales induites dans l'ectoblaste cultivé *in vitro*, il faut prendre en considération que l'élevage du chordo-mésoblaste en 'sandwiches' ectoblastiques peut modifier son pouvoir inducteur. Les expériences de ter Horst (1948) semblent plaider en faveur de cette hypothèse. En effet, cet auteur a parfois observé dans de telles conditions une évolution aberrante du chordo-mésoblaste, aboutissant occasionnellement à une neuralisation directe. Quoique je n'aie pas, moi-même, observé une différenciation anormale de reliquats d'inducteur laissés par mégarde dans certains de mes explantats, on ne peut exclure pour autant que le pouvoir inducteur du chordo-mésoblaste ne fût modifié. Cette hypothèse rendrait particulièrement bien compte de la formation exceptionnelle d'acrencéphale dans l'ectoblaste mis en contact pendant une courte durée avec le chordo-mésoblaste relativement très jeune, phénomène jamais rencontré lors de mes expériences de transplantations. Faute de preuves définitives, la question reste toutefois en suspens.

Pour conclure, les expériences présentes confirment pleinement et complètent les résultats de mon investigation précédente. Le chordo-mésoblaste est primitivement dépourvu de toute capacité d'induire du cerveau antérieur. Au moment où, dans une gastrula, ce matériel, en s'invaginant, se glisse sous le feuillet externe, il exerce d'emblée une action inductrice deutencéphalogène et spinale. Son faible pouvoir inducteur acrencéphalogène est une acquisition plus ou moins tardive qui ne s'affirme qu'au moment où le processus d'induction neurale est presque achevé chez l'embryon. Cependant la puissance inductrice deutencéphalogène et spinale prédomine toujours dans la région chordale de la voûte archentérique. Seul le chordo-mésoblaste âgé, et déjà évolué, est capable d'induire occasionnellement des complexes exclusivement acrencéphaliques; et ce phénomène n'est réalisable qu'en dissociant artificiellement les effets de l'action inductrice acrencéphalogène de ceux provoqués par le pouvoir inducteur deutencéphalogène et spinal, dont le chordo-mésoblaste est toujours doté. Ce dernier résultat peut être obtenu en interrompant le contact inducteur suffisamment tôt et au moment opportun. En effet, de nombreuses recherches (Toivonen, 1950, 1958; Toivonen & Saxén, 1955; Kuusi, 1951) indiquent que l'induction du cerveau antérieur est due aux substances chimiquement plus simples, plus diffu-

sibles et au poids moléculaire moindre que celles responsables de la formation du cerveau postérieur et de la moelle. Ces premières substances doivent donc agir sur l'ectoblaste plus rapidement que les secondes.

Il convient de relever que Toivonen, dans son travail récent (1958), en soumettant à une analyse critique les conclusion tirées par Nieuwkoop et Johnen de leurs expériences, arrive à une interprétation de l'induction neurale analogue à bien des égards à celle que je propose ici. Cependant cet auteur, en travaillant sur divers hétéro-inducteurs, n'a évidemment pas pu observer l'évolution progressive des capacités inductrices du chordo-mésoblaste, évolution que mes expériences démontrent clairement.

La culture *in vitro* exerce sur les structures neurales sous-induites, et dont l'orientation évolutive est encore très labile, une certaine influence, en les dirigeant vers la formation de l'acrencéphale.

RÉSUMÉ

Une seule espèce d'Urodèles, *Triturus alpestris*, a servi aux expériences. Les capacités inductrices de la zone chordale antérieure de la voûte archentérique sont étudiées *in vitro* à l'aide de la méthode de 'sandwiches' ectoblastiques. Le matériel inducteur est prélevé sur des gastrulas et des neurulas aux stades successifs de leur développement. La durée de l'induction (16 à 24 heures), suffisante pour provoquer une nette réaction dans l'ectoblaste, mais trop courte pour que le chordo-mésoblaste déploie totalement son activité inductrice régionale spécifique, fut déterminée d'après les résultats de mes expériences précédentes.

Cette période d'induction achevée, les deux feuillets du 'sandwich' sont décollés pour ôter l'inducteur, et ils sont élevés séparément dans la solution de Holtfreter. Aucune différence entre le pouvoir inducteur de la face dorsale et celui de la face ventrale de la voûte archentérique ne fut observée.

L'examen histologique des structures neurales fournies par les explantats confirme les résultats que j'ai obtenus précédemment, en exécutant des expériences analogues, mais à l'aide de transplantations sur l'embryon. Le pouvoir inducteur du chordo-mésoblaste se modifie considérablement au cours de la gastrulation et de la neurulation. Un faible pouvoir du chordo-mésoblaste d'induire du cerveau antérieur est une acquisition plus ou moins tardive, qui ne s'affirme qu'au moment où le processus d'induction neurale est déjà presque achevé chez l'embryon. Cependant, ce matériel embryonnaire est toujours doté d'une puissance inductrice deutencéphalogène et spinale prédominante. L'induction des complexes cérébro-sensoriels exclusivement acrencéphaliques par le chordo-mésoblaste ne devient possible qu'en dissociant artificiellement les effets de deux activités manifestées par l'inducteur.

La comparaison des résultats de mes expériences précédentes et actuelles révèle que l'induction du cerveau antérieur par le chordo-mésoblaste est plus facile à réaliser dans l'ectoblaste cultivé *in vitro* que dans les greffons implantés

sur la voûte archentérique et transplantés ensuite sur la face ventrale d'un autre embryon. Dans le premier cas, non seulement la fréquence des prestations acrencéphaliques est manifestement plus élevée, mais on peut déjà les obtenir à partir du chordo-mésoblaste plus jeune. La solution saline de Holtfreter exerce sur les structures neurales, issues d'une induction insuffisante, et dont l'orientation évolutive est encore labile, une faible action acrencéphalogène.

La conception de l'induction neurale émise par Nieuwkoop, et l'interprétation conforme aux vues de cet auteur que Johnen adopte pour expliquer les résultats de ses expériences, sont l'objet d'une révision détaillée.

SUMMARY

One species of Urodele, *Triturus alpestris*, was used. The inducing capacities of the anterior chordal zone of the archenteron roof were studied *in vitro* by the ectoblastic 'sandwich' method. The inducing material was taken from different stages of gastrulae and neurulae. The duration of induction (16–24 hours) had been determined by the results of the author's previous experiments: it was sufficient to provoke a clear reaction in the ectoblast, but too short for the chordomesoblast to exert fully its specific regional inductive capacity.

When the period of induction was at an end, the two layers of the 'sandwich' were separated to remove the inductor, and the layers were allowed to develop separately in Holtfreter's solution. No difference was found in the inductive capacities of the dorsal and ventral surfaces of the archenteron roof.

Histological examination of the neural structures produced by the explants confirmed the results obtained previously in analogous experiments involving transplants to the embryo. The inductive power of the chordomesoblast becomes considerably modified during gastrulation and neurulation. Its feeble power of inducing a forebrain is a more or less delayed acquisition, which only comes into action at the time when the process of neural induction is almost achieved in the embryo. It always possesses, however, a predominant power of inducing deutencephalon and spinal cord. The induction by the chordomesoblast of cerebro-sensory complexes which are exclusively acrencephalic becomes possible only when one dissociates the effects of the two activities manifested by the inductor.

A comparison of these results with those of the author's previous experiments shows that it is easier for the chordomesoblast to induce a forebrain in ectoblast cultivated *in vitro* than in grafts implanted on the archenteron roof and then transplanted to the ventral surface of another embryo. In the first case, not only are acrencephalic formations much more frequent, but one can obtain them with a younger chordomesoblast. The saline solution of Holtfreter has a feeble acrencephalogenic action on the neural structures which have resulted from an insufficient induction and which are still labile in the direction of their development.

The author reviews the conception of neural induction held by Nieuwkoop, and the similar interpretation adopted by Johnen to explain the results of her experiments.

TRAVAUX CITÉS

Bairati, A., & Tipoli, G. (1954). Ricerche morfologiche e istochimiche sulla glia del nervasse di Vertebrati. *Z. Zellforsch.* **39,** 392–413.

Barth, L. G. (1941). Neural differentiation without organizer. *J. exp. Zool.* **87,** 371–81.

Dalcq, A. (1946). L'Induction du prosencéphale. *Bull. Acad. Roy. Méd. Belg.* **11,** 98–115.

—— (1947). Sur l'induction de l'épiphyse et sa signification pour la morphogénèse du cerveau antérieur. *Arch. Portug. Sci. Biol.* **9,** 18–41.

—— & Lallier, R. (1948). Transplantations de territoires variés de la zone marginale médio-dorsale de la jeune gastrula chez le Triton. *Arch. Biol.* **59,** 267–378.

Damas, H. (1947). Effets de la suspension précoce du flux inducteur sur la détermination du neurectoblaste médullaire. *Arch. Biol.* **58,** 15–57.

Denis, H. (1957). Influence du facteur temps sur la détermination de la plaque neurale chez les Amphibiens. *Ann. Soc. zool. Belg.* **87,** 501–36.

Eyal-Giladi, H. (1954). Dynamic aspects of neural induction in Amphibia. *Arch. Biol.* **64,** 179–259.

Freedmann, B. (1953). Hyaluronidase effects on thionin-stained sections of brain. *Anat. Rec.* **115,** 265–70.

Gallera, J. (1947). Effets de la suspension précoce de l'induction normale sur la partie préchordale de la plaque neurale chez les Amphibiens. *Arch. Biol.* **58,** 221–64.

—— (1949). Transplantations de matériel inducteur céphalique du *Triton* prélevé durant la gastrulation et les premiers stades de la neurulation. *Arch. Anat. Strasbourg,* **32,** 121–72.

—— (1959). Le Facteur 'temps' dans l'action inductrice du chordo-mésoblaste et l'âge de l'ectoblaste réagissant. *J. Embryol. exp. Morph.* **7,** 487–511.

Hayashi, Y. (1958). The effects of pepsin and trypsin on the inductive ability of pentose nucleoprotein from guinea-pig liver. *Embryologia,* **4,** 33–53.

—— & Takata, K. (1958). Morphogenetic effects of subfractions of pentose nucleoprotein from the liver separated by means of ultracentrifugation. *Embryologia,* **4,** 149–60.

Hess, A. (1953). The ground substance of the central nervous system revealed by histochemical staining. *J. comp. Neurol.* **98,** 69–91.

—— (1955). The ground substance of the developing central nervous system. *J. comp. Neurol.* **102,** 65–75.

Hoessels, E. L. M. J. (1957). *Évolution de la plaque préchordale d'*Amblystoma mexicanum; *sa différenciation propre et sa puissance inductrice pendant la gastrulation.* Edit. J. Stegen, Maastricht.

Holtfreter, J. (1933). Der Einfluß von Wirtsalter und verschiedenen Organbezirke auf die Differenzierung von angelagerten Gastrulaektoderm. *Roux Arch. EntwMech. Org.* **127,** 619–775.

—— (1938). Differenzierungpotenzen isolierter Teile der Urodelengastrula. *Roux Arch. EntwMech. Organ.* **138,** 522–656.

—— (1943). Properties and functions of the surface coat in amphibian embryo. *J. exp. Zool.* **93,** 251–323.

—— (1944). Neural differentiation of ectoderm through exposure to saline solution. *J. exp. Zool.* **95,** 307–43.

—— (1947). Neural induction in explants which have passed through a sublethal cytolysis. *J. exp. Zool.* **106,** 197–222.

Hori, R., & Nieuwkoop, P. D. (1955). Induction phenomena with denaturated inductor. *Proc. Acad. Sci. Amst.* **58,** 266–72.

Horst, J. ter (1948). Differenzierungs- und Induktionsleistungen verschiedener Abschnitte der Medullarplatte und des Urdarmdaches von Triton im Kombinat. *Roux Arch. EntwMech. Organ.* **143,** 275–303.

Johnen, A. G. (1956). Experimental studies about the temporal relationships in the induction process. I, II. *Proc. Acad. Sci. Amst.* **59,** 554–61.

Karasaki, S. (1957). On the mechanism of the dorsalization in the ectoderm of *Triturus* gastrulae caused by precytolitic treatments. I, II. *Embryologia,* **3,** 317–41.

—— (1959). Electron microscopic studies on cytoplasmic structures of ectoderm cells of *Triturus* embryo during the early phase of differentiation. *Embryologia,* **4,** 247–72.

Kuusi, T. (1951). Über die chemische Natur der Induktionsstoffe mit besonderer Berücksichtigung der Rolle der Proteine und der Nukleinsäuren. *Ann. zool. Soc.* (Vanamo), **14,** 1–98.

LEHMANN, F. E. (1938). Regionale Verschiedenheiten des Organisators von Triton, insbesondere in der vorderen und hinteren Kopfregion, nachgewiesen durch phasenspezifische Erzeugung von lithiumbedingten und operativ bewirkten Regionaldefekten. *Roux Arch. EntwMech. Organ.* **138,** 106–59.

—— (1941). Die Sonderstellung des Zwischenhirns in der vergleichenden und experimentellen Embryologie. *Schweiz. med. Wschr.* **71,** 485–89.

—— (1945). *Einführung in die physiologische Embryologie.* Birhäuser.

NIEUWKOOP, P. D., *et al.* (1952). Activation and organization of the central nervous system in Amphibians. *J. exp. Zool.* **102,** 1–108.

—— & NIGTEVECHT, G. V. (1954). Neural activation and transformation in explants of competent ectoderm under the influence of fragments of anterior notochord in Urodeles. *J. Embryol. exp. Morph.* **2,** 175–93.

NIU, M. C., & TWITTY, V. C. (1953). The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. *Proc. nat. Acad. Sci., Wash.* **39,** 985–9.

OKADA, Y., & HAMA, T. (1943). Examination of regional differences in the inductive activity of the organizer by means of transplantation into ectodermal vesicles. *Proc. imp. Acad. Japan,* **19,** 48–53.

—— —— (1945). Prospective fate and inductive capacity of the dorsal lip of the blastopore of the *Triturus* gastrula. *Proc. imp. Acad. Japan,* **21,** 342–8.

—— & TAKAYA, H. (1942). Experimental investigation of regional differences in the inductive capacity of the organizer. *Proc. imp. Acad. Japan,* **18,** 505–13.

SALA, M. (1956). The character of the 'local activations' formed in sandwich explants of competent ectoderm containing neural formations induced by different cranio-caudal portions of archenteron roof. *Proc. Acad. Sci. Amst.* **59,** 661–7.

TAKAYA, H. (1953). On the loss of the notochord-forming potency in the prechordal plate of the *Triturus* gastrula. *Annot. zool. jap.* **26,** 202–7.

TOIVONEN, S. (1950). Stoffliche Induktoren. *Rev. suisse Zool.* **57,** 41–50.

—— (1958). The dependence of the cellular transformation of the competent ectoderm on temporal relationships in the induction process. *J. Embryol. exp. Morph.* **6,** 479–85.

—— & SAXÉN, L. (1955). Über die Induktion des Neuralrohres bei Trituruskeimen als simultane Leistung des Leber- und Knochenmarkgewebes von Meerschweinchen. *Ann. Acad. Sci. fenn. A IV Biologica,* **30,** 1–30.

WOELLWARTH, C. VON (1952). Die Induktionsstufen des Gehirns. *Roux Arch. EntwMech. Organ.* **145,** 582–668.

YAMADA, T. (1958). Embryonic Induction. In *A Symposium on the Chemical Basis of Development,* ed. W. McElroy & B. Glass, pp. 217–38. Baltimore: Johns Hopkins Univ. Press.

—— (1959). A progressive change in regional inductive effects of the bone-marrow caused by heat-treatment. *Embryologia,* **4,** 175–90.

EXPLICATIONS DE LA PLANCHE

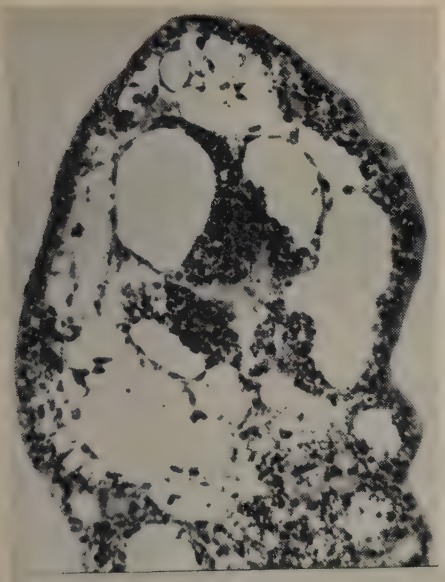
FIG. 1. L'inducteur utilisé fut prélevé sur une gastrula au stade du grand blastopore circulaire. De cette formation de caractère plutôt neuroïdal, des feuillets de cellules périphériques se sont détachés pour former des kystes latéraux. Dans la région centrale apparaissent des neurones bien différenciés délimitant de la substance blanche primitive. ×130.

FIG. 2. Une neurula à bourrelets médullaires a fourni l'inducteur. Un cerveau antérieur de forme irrégulière a engendré un œil petit mais correctement proportionné. A droite, on voit une belle placode olfactive. ×130.

FIG. 3. L'inducteur provient également d'une neurula à bourrelets médullaires. A la suite d'une compétition physiologique manifeste, l'ébauche oculaire s'est accaparée de la majeure partie du matériel disponible, provoquant une réduction passive du cerveau. Toute ébauche optique est constituée presque exclusivement par le feuillet sensoriel de la rétine. ×130.

FIG. 4. Une portion de l'inducteur est restée par mégarde attachée à l'ectoblaste. Ce matériel inducteur a alors évolué en tronçon chordal coudé (la coupe reproduite passe précisément par ce coude) et un petit amas de tissu musculaire. Ces formations adhèrent à l'ébauche oculaire qui a suscité la formation d'un petit cristallin (à gauche). ×130.

(*Manuscript received 30: xii: 59*)



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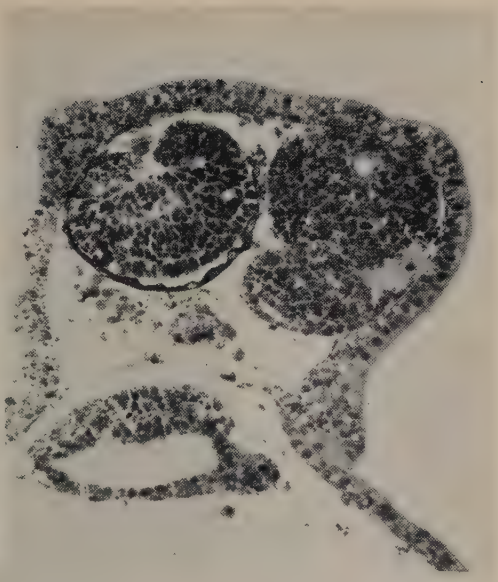

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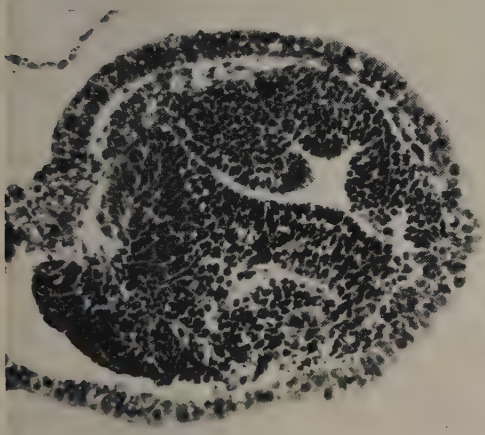

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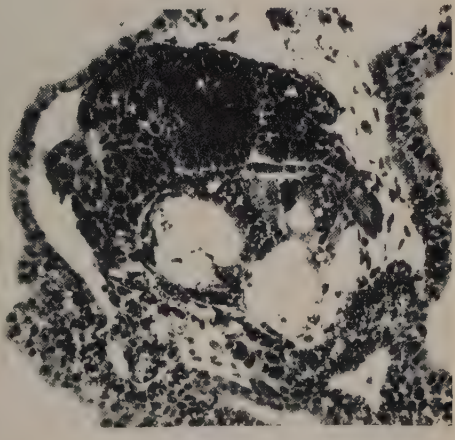

4

J. GALLERA

Internal Factors Influencing Normal and Compensatory Growth of the Axolotl Pronephros

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WITH ONE PLATE

INTRODUCTION

THE factors that influence compensatory growth of the pronephros in amphibian larvae may be either mechanical (hydrostatic) or, in a general sense, chemical. On the one hand, coelomic fluid, which enters the complex mass of pronephric tubules along the ciliated nephrostomes, must exert an intra-tubular fluid pressure. On the other hand, pronephroi bathed in blood of the post-cardinal sinus, receiving arterial blood from the dorsal aorta and transmitting coelomic fluid, could well be affected by chemical substances in these fluids.

In the present work an investigation was made of these two possible causal factors in *Ambystoma* larvae in order to discover whether pronephric growth promoting or inhibiting substances influence normal or compensatory growth *in vivo*, or whether hydrostatic influences are the more important.

It will be shown that there are no grounds for assuming the presence of specific stimulatory or inhibitory pronephric growth substances in young larvae, though the possibility cannot be ruled out in older stages, and that although the pronephric blastema cells will differentiate into underdeveloped tubules when they have no communication with the coelom, 'normal' tubular pattern and tubular hypertrophy (and probably hyperplasia) are determined by intra-tubular tension.

MATERIAL AND METHODS

All operations were performed on decapsulated embryos of *A. mexicanum* at Harrison stages 24–26 approximately (see Hamburger, 1950), when they were immersed in full-strength Holtfreter solution containing 0·2 per cent. sodium sulphadiazine (Fox, 1955). Recipients received a pronephric blastema graft from either the right- or the left-hand side of donors of the same age (one donor thus supplying two recipients), for there is no difference between the pronephroi of either side. Recipient and donor were arranged side by side on a gelatine base in

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a solid watch-glass, and, with instruments described by Hamburger (1950), a pronephric blastema (or some portion of it), together with its overlying ectoderm, was transferred from donor to host and inserted with the ectoderm lying uppermost into a surface wound prepared just behind and below the left presumptive forelimb region. Removal and grafting of the small whitish 'pulpy' pronephric blastema alone was unsatisfactory, for the tissue either disintegrated in the culture medium or stuck too tenaciously to the glass needle or failed to adhere permanently to the host. A small glass cover-slip delicately laid over the graft prevented movement and facilitated healing, which took place within 3–6 hours. Operated specimens were kept in the above-mentioned medium for 3 days and then they were transferred to glass crystallizing dishes (6 specimens to each dish) containing about 80 ml. of one-tenth-strength Holtfreter solution and 0·2 per cent. sodium sulphadiazine. Solutions were changed every 24 hours and the temperature during development varied between approximately 18° and 23° C.

Group I consisted of 7 control specimens each 10 mm. long, which were treated exactly like the operated ones but suffered neither unilateral pronephrectomy nor grafting. Group II is composed of 9 host specimens whose pronephroi were undisturbed, but each animal received graft tissue; the grafts in these specimens comprise Group II*a*. Group III comprises 8 specimens which were unilaterally pronephrectomized only (Howland, 1916, 1921; Fox, 1956). Group IV is composed of 10 host specimens which were unilaterally pronephrectomized like those in Group III, but each one received a pronephric graft. The grafts in these specimens comprise Group IV*a*. A few of the pronephrectomized specimens did not heal completely in the operated region so that the gut possessed a small opening to the exterior. Most of the grafts were situated behind the pronephric region, ventro-laterally to the lateral plate mesoderm, close to the coelom; all were entirely healed into the body tissue. Only those specimens which possessed graft tubules closed off from the coelom were employed in the main analysis. Graft tubules were thus isolated and probably were never in open communication with the coelomic fluid up to the time of examination.

Specimens were chosen at random from the many prepared, killed in Smith's fixative 10 days after operation when about 10 mm. long, paraffin-wax blocked and sectioned transversely at 10 μ. Other specimens were sectioned horizontally in order to measure nuclear length for the purpose of applying a correction to the pronephric nuclear population (Abercrombie, 1946). Four further specimens were transversely sectioned at 10 μ; two of them were like those in Group II (grafted specimens with undisturbed pronephroi) and the others like those in Group IV (grafted specimens unilaterally pronephrectomized), but with the difference that each tubular graft of these 4 specimens was open into the coelom by either one or two nephrostomial tubules.

Histological, sampling, and measuring techniques were identical to those used

in earlier papers (Fox, 1956, 1957, 1959). The level of statistical significance used throughout was $P<0{\cdot}05$.

RESULTS

(*a*) Comparison between pronephric components of Group I (control specimens with undisturbed pronephroi) and those of Group II (grafted specimens with undisturbed pronephroi).

Table 1

Means with standard errors of the various measurements on the control and operated groups of animals

		Grafted specimens with undisturbed pronephroi		*Unilateral pronephrectomies*	*Grafted unilaterally pronephrectomized specimens*	
	Controls Group I	*Pronephroi Group II*	*Grafts Group IIa*	*Pronephroi Group III*	*Pronephroi Group IV*	*Grafts Group IVa*
Number of larvae	7	9	9	8	10	10
Mean total length of larvae (mm.)	10·0 ±0·029	10·0 ±0·236	10·0 ±0·236	9·50 ±0·189	9·60 ±0·163	9·60 ±0·163
Mean nose-to-cloacal length of larvae (mm.)	6·29 ±0·070	5·99 ±0·166	5·99 ±0·166	5·71 ±0·159	5·81 ±0·101	5·81 ±0·101
Number of pronephroi (or pronephric grafts)	14	18	9	8	10	10
Mean ant.-post. length of pronephros (or pronephric graft) (mm.)	0·640 ±0·0112	0·603 ±0·0132	0·279 ±0·0257	0·681 ±0·0229	0·726 ±0·0186	0·410 ±0·0471
Mean nuclear population	1,506±56	1,544±59	491±92	1,562±48	1,739±107	664±89
Mean total volume of cells of pronephros (or grafts) (mm.^{3} $\times 10^{-3}$)	11·1 ±0·56	11·4 ±0·60	3·2 ±0·54	17·7 ±0·76	17·8 ±1·17	4·5 ±0·63
Mean total volume of lumen of pronephros (or graft) (mm.^{3} $\times 10^{-3}$)	9·0 ±0·48	9·7 ±0·85	1·2 ±0·44	26·6 ±7·38	30·2 ±2·91	1·1 ±0·17
Mean overall volume of pronephros (or graft) (mm.$^3 \times 10^{-3}$)	20·1 ±0·94	21·1 ±0·132	4·4 ±0·79	44·2 ±7·70	48·0 ±3·70	5·6 ±0·79
Mean tubule ratio vol. lumen/vol. tissue of pronephros (or graft)	0·8148 ±0·04021	0·8533 ±0·05334	0·3745 ±0·11723	1·4787 ±0·36596	1·7134 ±0·14025	0·2325 ±0·01435
Mean internal surface area of pronephric (or graft) tubules (mm.^{2})	0·6559 ±0·02864	0·6146 ±0·03461	0·0881 ±0·01647	0·8263 ±0·07272	0·9517 ±0·04119	0·1145 ±0·01600
Mean calculated individual cell volume of pronephros (or graft) (μ^3)	7,371 ±187	7,332 ±181	6,953 ±287	11,348 ±505	10,253 ±285	6,807 ±268
Mean nuclear length at right angles to larval transverse section (250)	11·947 ±0·1839	12·388 ±0·1803	12·137 ±0·1720	12·274 ±0·2269	12·517 ±0·2333	12·335 ±0·2148

Means of the total length and of the nose-to-cloacal length of the two groups of larvae and of the various components of the pronephric systems do not differ significantly (Tables 1, 2; Plate, figs. A, B) except for the means of the antero-posterior pronephric length and of the diameter of the anterior and posterior nephrostome bases, which are significantly lower in Group II by 6 per cent. and 9 per cent. respectively.

TABLE 2

Means with standard errors of the various measurements on the control and operated groups of animals

		Grafted specimens with undisturbed pronephroi		*Unilateral pronephrectomies*	*Grafted unilaterally pronephrectomized specimens*	
	Controls Group I	*Pronephroi Group II*	*Grafts Group IIa*	*Pronephroi Group III*	*Pronephroi Group IV*	*Grafts Group IVa*
Number of larvae	6	6	9	8	10	10
Mean total length of larvae (mm.)	10·0 ±0·033	10·0 ±0·254	10·0 ±0·236	9·50 ±0·189	9·60 ±0·163	9·60 ±0·163
Mean nose-to-cloacal length of larvae (mm.)	6·27 ±0·080	5·89 ±0·215	5·99 ±0·166	5·71 ±0·159	5·81 ±0·101	5·91 ±0·101
Number of pronephroi (or grafts)	12	12	9	8	10	10
Mean diameter of ant. nephrostome base (mm.)	0·0600 ±0·00303 (12)	0·0542 ±0·00193 (12)	..	0·0588 ±0·00295 (8)	0·0640 ±0·00341 (10)	..
Mean diameter of post. nephrostome base (mm.)	0·0600 ±0·00275 (12)	0·0550 ±0·00151 (12)	..	0·0637 ±0·00565 (8)	0·0700 ±0·00683 (10)	..
Mean diameter of anterior and posterior nephrostome bases (mm.)	0·0600 ±0·00199 (24)	0·0546 ±0·00120 (24)	..	0·0612 ±0·00315 (16)	0·0670 ±0·00378 (20)	..

One control (Group I) and 2 specimens of Group II possess pronephroi each with 3 nephrostomes (see Field, Plate VIII, fig. 57, 1891), and one further specimen of Group II had a pronephros whose anterior nephrostome was divided into two. These pronephroi were not otherwise 'abnormal' and only the abnormal nephrostomes were omitted from the analysis.

(*b*) Comparison between host pronephroi in specimens of Group II (grafted specimens with paired undisturbed pronephroi) and their grafts (Group II*a*).

Only two components from each group were compared; other comparisons between whole undisturbed pronephroi and a mere portion of pronephric tissue are not applicable. Means of the calculated individual cell volumes of host pronephroi and grafts do not differ significantly, although there is a smaller cell volume in the latter. The second measurement employed was the relationship lumen volume/tissue volume, henceforth termed the 'tubule ratio'. Use of the tubule ratio is a method of quantifying an obvious morphological feature of the graft tissue. Graft tubules have thickish, somewhat vacuolated walls, the lumina are attenuated and considerably reduced in volume and show no typically expanded 'normal' tubular pattern (Plate, figs. C, D, I, L). This abnormal appearance is typical of any graft (whichever group is considered) when not in open communication with the coelom. Only one anomalous graft had a large expanded tubule which ended at the surface of the body, and here its condition encouraged the view that at some time it was open to the exterior. The graft mean tubule ratio (Group II*a*) is significantly lower than that of the pronephroi (Group II) by 56 per cent. (Table 1).

(*c*) Comparison between pronephroi in specimens of Group I (controls with paired undisturbed pronephroi) and those of Group III (specimens unilaterally pronephrectomized without grafts).

Pronephric components of Group III showed a significant superiority over those in Group I by the stated percentages in the means of the following measurements: total lumen volume (196 %); overall pronephric volume (tissue volume and lumen volume together) (120 %); tubule ratio (81 %); total cell volume (60 %); calculated individual cell volume (54 %); internal surface area of the tubules (26 %) (see Plate, figs. A, E, F). Means of the total length and of the nose-to-cloacal length of the larvae were significantly lower in Group III by 5 per cent. and 9 per cent. respectively (Table 1). Howland (1921) has shown that unilateral pronephrectomy may inhibit general growth and a similar result occurred in the other unilaterally pronephrectomized specimens (Group IV). Retardation did not occur in similar circumstances in *Triturus cristatus carnifex* (Fox, 1956).

No significant differences were found between the means of the following measurements: antero-posterior pronephric length (6·4 per cent. superiority in Group III over Group I); diameter of the anterior and of the posterior nephrostomial bases, and of the total nephrostome bases; mean pronephric nuclear population (3·7 per cent. superiority in Group III over Group I). These results may be compared with those of previous work (Fox, 1956, 1957). In unilaterally pronephrectomized *T. cristatus carnifex* (killed 10–15 days after a stage equivalent to Harrison stage 24), and operated specimens of *T. helveticus* with unilateral duct blockage (killed 20 days after operation at stages 21–23, Glaesner, 1925), there were the following percentage compensatory responses compared with controls (*T. helveticus* is shown in brackets). Lumen volume 100 (127); overall pronephric volume 52 (94); tubule internal surface area 49 (63); total cell volume 34 (78); nuclear population 17 (41); calculated individual cell volume 15 (22); antero-posterior pronephric length 14 (20).

Calculation from the data for *T. helveticus* shows the tubule ratio of hypertrophied (compensated) pronephroi to have a significant superiority of 31 per cent. over the controls; i.e. 0·612±0·0610 (compensated pronephroi), 0·466± 0·0380 (controls). For *T. cristatus carnifex* the tubule ratio of compensated pronephroi 10 days after a stage equivalent to Harrison stage 24 showed a significant superiority of 32 per cent. over controls; i.e. 0·393±0·0280 (unilaterally pronephrectomized specimens), 0·298±0·0150 (controls), and 15–17 days after a stage equivalent to Harrison stage 24 the tubule ratio showed a significant superiority of 49·6 per cent. over controls; i.e. 0·758±0·0856 (unilaterally pronephrectomized specimens), 0·506±0·0141 (controls).

Howland (1921) in one *Ambystoma* specimen fixed 9 days after unilateral pronephrectomy at Harrison stages 30–32 found the total cell volume to increase by 63 per cent. when compared with a control specimen, and other increases were nuclear hyperplasia (16 %), tubule length (21 %), internal surface area

of tubules (over 100 %), and in addition there was actual cell-volume hypertrophy.

The results obtained in *Ambystoma* in this present work, with the exception of the nuclear population and pronephric length, are generally similar to those in the *Triturus* species, and confirm some of the results of Howland.

(*d*) Comparison between pronephroi of Group I (controls with paired undisturbed pronephroi) and host pronephroi of Group IV (grafted unilaterally pronephrectomized specimens).

Means of the pronephric components of Group IV showed a significant superiority over those in Group I in total lumen volume (236 per cent.); overall pronephric volume (139 %); tubule ratio (110 %); total cell volume (60 %); internal surface area of tubules (45 %); calculated individual cell volume (39 %); nuclear population (15·5 %); antero-posterior pronephric length (13 %) (see Plate, figs. A, G, H, K). Mean nose-to-cloacal length was significantly lower in Group IV by 8 per cent. and mean total larval length, though not significantly so, was lower by 4 per cent.

The means of the diameters of the anterior and of the posterior nephrostomial tubule bases and of all the nephrostomes together though showing percentage superiorities in Group IV over Group I are not significantly different (Tables 1, 2).

(*e*) Comparison between pronephroi in Group III (specimens unilaterally pronephrectomized only) and those in Group IV (grafted unilaterally pronephrectomized specimens).

No significant differences were found between the pronephric component measurements of the two groups (Tables 1, 2; Plate, figs. E, F, G, H, K).

(*f*) Comparison between host pronephroi in grafted unilaterally pronephrectomized specimens (Group IV) and their grafts (Group IV*a*).

Means of the individual cell volume and of the tubule ratio show a significant superiority in Group IV over Group IV*a* by 35 per cent. and 87 per cent. respectively (Table 1).

(*g*) Comparison between grafts in specimens with paired undisturbed pronephroi (Group II*a*) and grafts in specimens unilaterally pronephrectomized (Group IV*a*).

No significant differences were found between the means of the individual cell volumes and of the tubule ratios. Although not significant in both groups of grafts, there is a similar lower mean individual cell volume of 5·7 per cent. (Group II*a*) and 7·6 per cent. (Group IV*a*) when compared with controls (Table 1; Plate, figs. C, D, I).

DISCUSSION

In the larval *Ambystoma* population investigated, pronephric graft tissue, which presumably contained some damaged cells when transferred to a recipient, does not influence growth of undisturbed pronephroi, except for changes in

pronephric length and in the relatively unimportant nephrostomial base diameter. Nor does the graft influence pronephric compensatory growth, for compensatory hypertrophy takes place when a specimen is unilaterally pronephrectomized irrespective of whether graft tissue is present or not. Failure in this work to obtain significant increases in the pronephric nuclear population and pronephric antero-posterior length in non-grafted unilaterally pronephrectomized specimens may be an example of the variability of these responses, for compensatory hyperplasia and increase in pronephros length can and normally do take place in urodeles including *Ambystoma* (Howland, 1921) so treated, and both components show a trend in this direction in the present work. The results may be compared with those in *T. helveticus* (Fox, 1957), where it was found that pronephric compensatory hypertrophy (and hyperplasia) ensued in an unblocked functional pronephros when the duct of its partner is blocked, though the blocked pronephros, except for some stretching of the tubule walls, appeared undamaged. A pronephros thus compensates in the presence of a blocked but undamaged partner, and the pronephroi do not inhibit one another's growth—a result which does not support the theory of autoregulation of growth by homologous organs (see Weiss, 1952, 1953, 1955). The evidence in this present work supports the view that it is unlikely that products of normal or of damaged homologous cells influence pronephros cell size or tubule lumen size.

The grafts in unilaterally pronephrectomized specimens, like grafts in specimens with paired undisturbed pronephroi, were situated close to the coelom in intimate association with blood sinuses. If a raised concentration of circulating pronephric growth-promoting substances, however produced, elicited pronephric hypertrophy and hyperplasia, then it may be assumed that there would be a difference in concentration at some time between these substances in grafted unilaterally pronephrectomized specimens possessing a single hypertrophied pronephros, and in grafted specimens with undisturbed pronephroi. Yet the graft tubules in the two groups are similar in tubule appearance and in individual cell volume. Grafts in the two groups do, however, differ in cell number and in total volume. Nevertheless, the means of the total nuclear population, cell volume, lumen volume, and overall volume, were not found to differ significantly. Yet it is impossible to know exactly how much pronephric tissue has been transferred to a recipient, and these numerical differences may be due to the difference in the amount actually transferred, or to a real increase in mitosis (hyperplasia) of graft tissue of Group IV*a*. The presence of circulating pronephric growth substances which would influence individual cell volume and lumen volume is therefore not supported in this work, but the results do not discount the possibility that an increase in the rate of mitosis has taken place in the grafts of Group IV*a* and that this increase has been influenced by them.

A difference of major importance between grafts and any pronephros left *in situ* is that graft tubules are not in open communication with the coelomic

fluid. A pronephric blastema graft will differentiate into tubules although it is isolated from the coelom, but the lumina are small and the tubules fail to develop into normal functionally expanded structures. Their condition is similar to that of a pronephric duct remaining after unilateral pronephrectomy, or that portion of a duct distal to a lesion, for the duct is in a collapsed state. Among the collection of experimental animals were 4 specimens each with a graft in the usual ventrolateral position behind the pronephros region, and with an open communication to the coelom by either one or two ciliated nephrostomes (Plate, fig. J). These 'open' grafts differed from the blind isolated ones in that their tubules were to a greater or lesser extent expanded (like blocked pronephroi with nephrostome inlets but having no exit) (Fox, 1957). Tubule ratios of these grafts were: 3·467, 1·180, 0·740, and 0·446; all are higher than the means of the tubule ratios of isolated graft tubules in the two groups (II*a* and IV*a*) previously considered. It is clear that intra-tubular fluid pressure, exerted by coelomic fluid which enters by the ciliated nephrostomial tubules, maintained the graft tubules in an expanded state.

The presence of coelomic fluid directed into the pronephric tubules by nephrostomial cilia and exerting an intra-tubular fluid tension would seem to be essential for the maintenance of normal or hypertrophied tubule shape, and to some extent it influences cell size.

Pronephric grafts in either a compensating or non-compensating environment are extremely similar in the appearance of the tubules, and though they differ in cell numbers and total volume, these differences are not significant. In addition the mean individual cell volumes are the same, so it is likely that the mitotic rates of these graft tissues are similar. Yet in pronephric compensation there is usually hyperplasia, and as there is no evidence for the view that cell volume and lumen volume hypertrophy are initiated by pronephric growth-promoting substances, then intra-tubular tension may be the main causal factor eliciting compensatory nuclear proliferation also. This opinion supports the more cautious conclusion reached for the cause of pronephric hyperplasia in *T. helveticus* larvae each one possessing a blocked and an unblocked hyperplastic pronephros (Fox, 1957), and for the striking results in the paired pronephroi of *T. helveticus* grown in distilled water (Fox, 1959).

Animals unilaterally pronephrectomized were practically the same in size and shape as controls with paired undisturbed pronephroi. The external surface areas of these animals are generally similar and as the properties of the skin presumably do not differ, similar quantities of fluid will enter these larvae when they develop in media of the same kind. Because both types of larvae appear similar, then approximately the same quantity of fluid must have been evacuated from animals with either a single pronephros or a pair. Intra-tubular tension must therefore be higher in a single remaining pronephros than in either of the paired pronephroi, and it is likely that this super-normal intra-tubular fluid tension elicits all the recognized compensatory pronephric growth phenomena.

SUMMARY

1. The purpose of the experiments described in this paper was to distinguish between mechanical (hydrostatic) and chemical influences upon normal or compensatory growth of the pronephros in the larval axolotl (Harrison stages 24–26).

2. Measurements were made on pronephroi and pronephric grafts from the following groups: (*a*) control specimens with undisturbed pronephroi; (*b*) specimens with paired undisturbed pronephroi and grafts; (*c*) specimens unilaterally pronephrectomized; (*d*) specimens unilaterally pronephrectomized and containing grafts.

3. Analysis of the measurements shows that hydrostatic pressure within pronephric tubules is the main factor responsible for maintaining size and shape in both normal and compensatory growth.

RÉSUMÉ

Action de facteurs internes sur la croissance normale et compensatrice du pronéphros de l'Axolotl

1. Les expériences décrites dans cet article ont eu pour but de distinguer entre les influences chimiques et les influences mécaniques (hydrostatiques) qui s'exercent sur la croissance normale et compensatrice du pronéphros de la larve d'Axolotl.

2. On a fait des mesures sur les pronéphros et les greffes de pronéphros des groupes suivants: (*a*) spécimens témoins avec pronéphros intacts; (*b*) spécimens avec pronéphros intacts et greffons de pronéphros; (*c*) spécimens pronéphrectomisés unilatéralement; (*d*) spécimens pronéphrectomisés unilatéralement et contenant des greffons.

3. L'étude des mesures montre que la pression hydrostatique à l'intérieur des tubes du pronéphros est le principal facteur responsable du maintien de la taille et de la forme dans la croissance normale aussi bien que compensatrice.

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REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microscope sections. *Anat. Rec.* **94**, 239–47.

Field, H. H. (1891). The development of the pronephros and segmental duct in Amphibia. *Bull. Mus. comp. Zool. Harv.* **21**, 201–340.

Fox, H. (1955). The effect of sodium sulphadiazine on the nuclear population of the pronephros in *T. cristatus karelinii* (Strauch). *Exp. Cell Res.* **8**, 250–1.

—— (1956). Compensation in the remaining pronephros of *Triturus* after unilateral pronephrectomy. *J. Embryol. exp. Morph.* **4**, 139–51.

—— (1957). The effect of unilateral blockage of a pronephric duct upon the development of the pronephroi in *Triturus helveticus*. *J. Embryol. exp. Morph.* **5**, 274–82.

—— (1959). A study of environmental influences on the size of the pronephros and related structures in *Triturus helveticus*. *J. Embryol. exp. Morph.* **7**, 257–63.

Glaesner, L. (1925). *Normentafeln zur Entwicklungsgeschichte der Wirbeltiere* (Molge vulgaris). Jena: Gustav Fischer.

Hamburger, V. (1950). *A Manual of Experimental Embryology*. Chicago University Press.

Howland, R. B. (1916). On the removal of the pronephros of amphibian embryos. *Proc. nat. Acad. Sci. Wash.* **2**, 231–4.

—— (1921). Experiments on the effects of removal of the pronephros of *Amblystoma punctatum*. *J. exp. Zool.* **32**, 355–95.

Weiss, P. (1952). Self-regulation of organ growth by its own products. *Science*, **115**, 487–8.

—— (1953). Some introductory remarks on the cellular basis of differentiation. *J. Embryol. exp. Morph.* **1**, 181–211.

—— (1955). Specificity in growth control. In *Biological Specificity and Growth*, ed. E. G. Butler, pp. 195–206. Princeton University Press.

EXPLANATION OF PLATE

All sections are transverse, except figs. K and L which are horizontal and 10 μ thick.

Abbreviations: *c.p.t.*, control pronephric tubule; *co.*, coelom; *comp. g.p.t.*, hypertrophied pronephric tubule in grafted unilaterally pronephrectomized specimen (Group IV); *comp. p.t.*, hypertrophied pronephric tubule in specimen only unilaterally pronephrectomized (Group III); *g.*, gut; *g.p.t.*, pronephric tubule in grafted specimen with paired undisturbed pronephroi (Group II); *glom.*, glomus; *grt.*, pronephric graft; *n.c.*, nerve-cord; *neph.*, nephrostome; *not.*, notochord; *p.d.*, pronephric duct.

Fig. A. Control specimen (Group I). Section 2·41 mm. from the anterior tip of head. Left-hand side pronephros 0·62 mm. and right 0·59 mm. long. Left pronephros 0·31 mm. and right 0·32 mm. from anterior end of pronephros. Specimen 10 mm. long, nose-to-cloaca 6·24 mm.

Fig. B. Grafted specimens with undisturbed pronephroi (Group II). Section 2·37 mm. from anterior tip of head. Left-hand side pronephros 0·66 mm. and right 0·60 mm. long. Left pronephros 0·37 mm. and right 0·42 mm. from anterior end of pronephros. Specimen 10 mm. long, nose-to-cloaca 5·71 mm.

Fig. C. Same specimen as in fig. B. Section through pronephric graft 3·01 mm. from anterior tip of head. Graft 0·32 mm. long, section is 0·11 mm. from its anterior end.

Fig. D. Grafted specimen with undisturbed pronephroi (Group II). Section through pronephric graft 2·62 mm. from anterior tip of head. Graft 0·30 mm. long, section is 0·17 mm. from its anterior end. Specimen 9 mm. long, nose-to-cloaca 5·27 mm.

Fig. E. Non-grafted unilaterally pronephrectomized specimen (Group III). Section 2·16 mm. from anterior tip of head. Remaining hypertrophied pronephros 0·66 mm. long, section is 0·31 mm. from its anterior end. Specimen 9 mm. long, nose-to-cloaca 5·50 mm.

Fig. F. Non-grafted unilaterally pronephrectomized specimen (Group III). Section 2·00 mm. from anterior tip of head. Remaining hypertrophied pronephros 0·79 mm. long, section is 0·32 mm. from its anterior end. Specimen 10 mm. long, nose-to-cloaca 5·45 mm.

Fig. G. Grafted unilaterally pronephrectomized specimen (Group IV). Section 2·25 mm. from anterior tip of head. Remaining hypertrophied pronephros 0·72 mm. long, section is 0·52 mm. from its anterior end. Specimen 10 mm. long, nose-to-cloaca 5·71 mm.

Fig. H. Grafted unilaterally pronephrectomized specimen (Group IV). Section 2·15 mm. from anterior tip of head. Remaining hypertrophied pronephros 0·73 mm. long, section is 0·38 mm. from its anterior end. Specimen 9 mm. long, nose-to-cloaca 5·64 mm.

Fig. I. Same specimen as in fig. G. Section through pronephric graft 2·47 mm. from anterior tip of head. Graft 0·37 mm. long, section is 0·19 mm. from its anterior end.

Fig. J. Grafted specimen with undisturbed pronephroi (as in Group II). Section through pronephric graft (with open coelomic nephrostome) is 3·41 mm. from anterior tip of head. Graft 0·47 mm. long, section is 0·41 mm. from its anterior end. Specimen 10 mm. long, nose-to-cloaca 6·80 mm.

Fig. K. Grafted unilaterally pronephrectomized specimen (as in Group IV). Hypertrophied pronephros from horizontal aspect. Section is 1·16 mm. from the ventral surface. Specimen 10 mm. long.

Fig. L. Specimen as in fig. K. Pronephric graft from horizontal aspect. Section is 0·55 mm. from the ventral surface.


A
n.c.
not.
c.p.t.
co.
g.
B
n.c.
not.
glom.
g.p.t.
g.
co.
C
n.c.
not.
p.d.
co.
g.
grt.
D
n.c.
not.
p.d.
g.
co.
grt.
E
n.c.
not.
comp.
p.t.
co.
F
n.c.
not.
comp.
p.t.
co.
G
n.c.
not.
glom.
comp.
g.p.t.
g.
co.
H
n.c.
not.
glom.
comp.
g.p.t.
co.
I
n.c.
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p.d.
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J
not.
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K
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L
grt.
grt.
g.
1mm.

H. FOX

The Developmental Capacity of Nuclei Taken from Differentiating Endoderm Cells of *Xenopus Laevis*

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INTRODUCTION

An important question concerning embryonic differentiation is whether the nuclei of somatic cells in different parts of an embryo come to differ genetically from each other during development. It has become possible to investigate this matter since King & Briggs (1955) have shown that nuclear transplantation is a satisfactory technique for testing the developmental potentialities of embryonic nuclei. These authors (1957, 1960) have used *Rana pipiens* for transplantation experiments with endoderm nuclei, and have found that these nuclei become progressively limited in their developmental capacity after the late blastula stage.

This paper describes some similar experiments carried out with endoderm nuclei of *Xenopus laevis*. The general conclusion that nuclei change as development proceeds is confirmed; there are, however, considerable differences between *Rana* and *Xenopus* in the rate and time of onset of nuclear changes. These differences make it easier to understand the significance of nuclear differentiation during embryonic development.

TECHNIQUE, DONOR NUCLEI, AND RECIPIENT EGGS

The technique used in these experiments with *Xenopus* has been modified from that of Briggs & King (1953) and is described elsewhere together with the method of culturing transplant-embryos (Elsdale, Gurdon, & Fischberg, 1960).

Donor cells have been prepared by dissecting out part of the endoderm with the help of needles. Cells are disaggregated by placing the isolated tissue in Barth–Versene solution for between 10 and 20 minutes (Gurdon, 1960*b*); they are then transferred to standard Barth solution (Barth & Barth, 1959) and kept until required for transplantation.

The developmental stages of donor embryos have been identified according to Nieuwkoop & Faber's (1956) normal table for *X. laevis*. All the experiments described below have been carried out with vegetal or endoderm donor cells.

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In blastulae and gastrulae donor cells were taken from the centre of the vegetal cell-mass, and in later stages from the floor of the gut lumen. In hatched tadpoles, cells were taken from the floor of the anterior part of the gut; these cells are rather larger and more easily disaggregated than those forming the outer wall of the gut. In hatched tadpoles the presumptive germ-cells do not lie in that part of the gut from which donor cells were taken.

Proof that the nuclei of a transplant-embryo have all been derived from the injected nucleus alone is provided by the use of marked donor nuclei. These are diploid and were obtained from the 1-nucleolated strain of *Xenopus* (Elsdale, Fischberg, & Smith, 1958). The presence of the nuclear marker can be recognized in a squash preparation of part of the donor embryo, since the number of nucleoli per nucleus can be quickly counted under the phase-contrast microscope (Elsdale, Gurdon, & Fischberg, 1960). Since marked donor nuclei have been injected into unmarked and enucleated recipient eggs, all transplant-embryos should have marked nuclei (i.e. with one nucleolus). Transplant-embryos are sometimes obtained which have two or more nucleoli in each nucleus. If the embryo's ploidy and nucleolar number is known, the origin of its nuclei is at once evident (Elsdale, Gurdon, & Fischberg, 1960). Tetraploids resulting from the doubling of the injected nucleus and triploids resulting from the participation of the egg nucleus both have 2-nucleolated nuclei, but are troublesome to distinguish by the number of chromosomes. For this reason, and because the tetraploid condition appears to have no effect upon the embryonic development of transplant-embryos (Gurdon, 1959), only 1-nucleolated diploids have been counted in the results below.

Recipient eggs were obtained by injecting frogs with gonadotropic hormone. Normal diploid frogs, which did not therefore carry the nuclear marker, were used. These frogs had either been kept in the laboratory since they were imported, or else they had been reared in the laboratory from imported frogs. The quality of eggs laid by recently imported frogs does not seem to differ consistently from that of frogs reared in the laboratory.

FACTORS AFFECTING TRANSPLANT-EMBRYO DEVELOPMENT

The way in which transplant-embryos develop is determined by many different factors, which fall into the four categories below. The effects of the last three must be known before it is possible to relate transplant-embryo development to the first. They are: (*a*) innate qualities of donor nuclei; (*b*) technical damage to donor nuclei; (*c*) innate qualities of recipient eggs; (*d*) technical damage to recipient eggs. Study of variation in the last three of these has shown that they may affect transplant-embryo development in only two ways (Gurdon, 1960*b*). The first concerns variation in the degree of donor-cell distortion, and the second concerns variation in egg quality (i.e. in the ability of eggs to recover from technical interference).

When donor cells of a similar volume are transplanted with different sized pipettes, this varies the extent to which the donor cell-wall is disrupted. It is found that the degree of donor-cell distortion is directly related to the proportion of total transplantations which form regular late blastulae (Gurdon, 1960*b*). It seems that, if the donor cell-wall is insufficiently broken, the injected nucleus is unable to combine satisfactorily with the recipient egg cytoplasm. Though the degree of donor-cell distortion has been kept constant as far as possible, small variations in this respect are unavoidable and can cause a variation of up to 20 per cent. in the proportion of late blastulae formed. For this reason it is best to base the main conclusions from transplantation experiments on the development of transplant-embryos expressed as a proportion of regular late blastulae, and not as a proportion of total transplantations.

When nuclei from the same donor embryo are transplanted (with no variation in technique) into eggs of different frogs, it was found that the development of transplant-embryos was appreciably more normal with the recipient eggs of some frogs than with those of others (Gurdon, 1960*b*). A test in which fertilized eggs were treated just as for nuclear transplantation, except that no nucleus was injected, showed that the standard transplantation treatment may result in abnormal development with the eggs of some frogs, but may cause no harm to those of others. This demonstrates that the eggs of different frogs vary considerably in their ability to withstand the experimental manipulations to which they are subjected. It is unfortunately not possible to recognize the quality of recipient eggs other than by the development to which they give rise. This makes it necessary to do control experiments using donor nuclei of known developmental potentialities, such as undifferentiated blastula nuclei. Control transplantations show in which experiments egg quality was good; only these 'selected' experiments can be regarded as giving a *clear* indication of innate nuclear qualities. The quality of recipient eggs is usually consistent throughout an ovulation, but it sometimes happens that eggs deteriorate in quality when a frog has already been laying for several hours. For this reason control transplantations are if possible carried out at the beginning *and* end of each experiment.

Experiments published elsewhere (Gurdon 1960*b*) have shown that abnormal development of transplant-embryos beyond the blastula stage cannot be caused by any part of the standard technical treatment of donor nuclei. If donor nuclei from several normal blastulae are transplanted into the eggs of one frog, they give very similar results (Text-fig. 4B). This demonstrates that blastula nuclei do not differ in their capacity to give normal transplant-embryo development, and are therefore suitable for controls with which to test the quality of recipient eggs. It is thus evident that transplantation experiments can be analysed in such a way that the only variable affecting transplant-embryo development is the innate quality of donor nuclei.

RESULTS

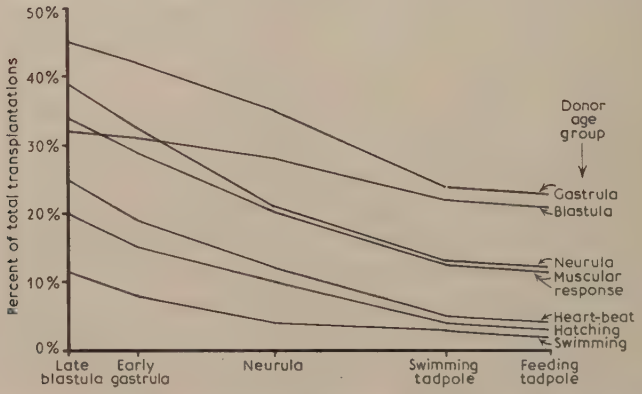
Table 1 is a summary of all experiments with endoderm nuclei carried out since the technique became standardized; it includes only diploid transplant-embryos from first-transfer experiments and does not contain the results of

TABLE 1

Total results with endoderm nuclei

The percentages represent transplant-embryo survival in terms of total transplantations for each donor

Donor embryo age group and normal stage numbers of Nieuwkoop (1956)	*Total trans-plantations*	*Stages of transplant-embryo development*				
		Late blastula	*Early gastrula*	*Late gastrula and neural folds*	*Swimming tadpole*	*Normal feeding tadpole*
Blastulae (7–9)	533 100%	172 32%	163 31%	147 28%	116 22%	109 21%
Gastrulae (10–13)	1,014 100%	452 45%	421 42%	354 35%	242 24%	229 23%
Neurulae (14–22)	469 100%	182 39%	135 29%	99 21%	58 13%	51 12%
Muscular response post-neurulae (23–26)	236 100%	77 34%	65 29%	47 21%	29 13%	26 12%
Heart-beat tadpoles (29–34)	282 100%	72 25%	54 19%	34 12%	15 5%	12 4%
Hatching tadpoles (35–37)	471 100%	95 20%	70 15%	44 10%	20 4%	13 3%
Swimming tadpoles (39–41)	681 100%	77 11·5%	54 8%	27 4%	20 3%	13 2%



TEXT-FIG. 1. Survival curves for *all* experiments (figures from Table 1). Each curve shows the survival of transplant-embryos for one donor age group. Within each donor group, survival is expressed as a percentage of total transplantations

serial transfers. In the survival curves of Text-fig. 1 there is an appreciable mortality among transplant-embryos from all donors; it happens that a higher proportion of late blastulae was obtained from gastrula and neurula donors than from blastula donors, but this difference is not significant. Apart from this

TABLE 2

Selected experiments only

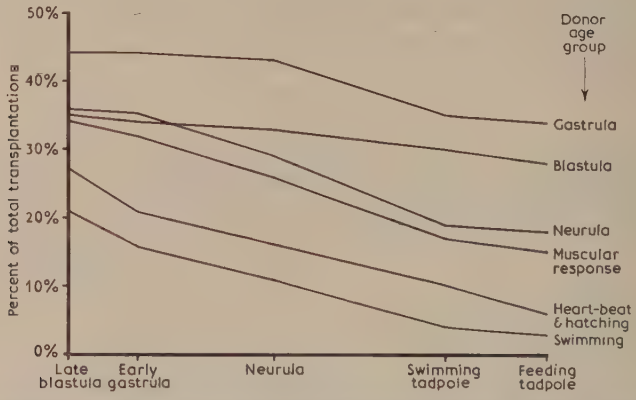
The percentages in the middle of each square represent transplant-embryo survival in terms of total transplantations; the percentages at the bottom of each square indicate transplant-embryo survival in terms of the number of late blastulae for that donor stage

Donor embryo age group and normal stage numbers of Nieuwkoop (1956)	*Total trans-plantations*	*Stages of transplant-embryo development*				
		Late blastula	*Early gastrula*	*Late gastrula and neural folds*	*Swimming tadpole*	*Normal feeding tadpole*
Blastulae (7–9)	327 100%	113 35% 100%	110 34% 98%	106 33% 94%	96 30% 85%	91 28% 81%
Gastrulae (10–13)	502 100%	221 44% 100%	220 44% 100%	216 43% 98%	175 35% 79%	169 34% 77%
Neurulae (14–22)	140 100%	50 36% 100%	48 35% 96%	40 29% 79%	27 19% 53%	26 18% 52%
Muscular response post-neurulae (23–26)	152 100%	53 34% 100%	50 32% 94%	40 26% 75%	25 17% 47%	22 15% 41%
Heart-beat and hatching tadpoles (29–34)	174 100%	47 27% 100%	36 21% 76%	28 16% 59%	17 10% 36%	13 6% 27%
Swimming tadpoles (39–41)	436 100%	89 21% 100%	71 16% 80%	48 11% 54%	17 4% 19%	13 3% 15%

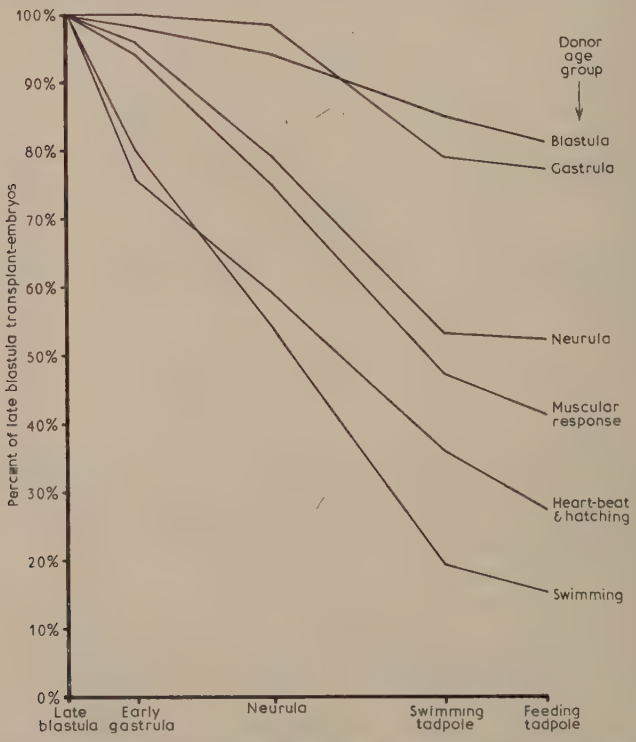
it can be seen that the older the donor embryos, the smaller is the proportion of late blastulae and normal tadpoles obtained from their nuclei. It is not profitable to examine these general trends in any detail, since they include experiments done on bad recipient eggs, many of which developed abnormally irrespective of the type of injected nuclei.

Table 2 contains only the results of 'selected' experiments, in which the recipient eggs were of good quality. There is no clear-cut way of classifying recipient eggs as good or bad, since all degrees of egg quality are found. The best that can be done is to group together all those experiments in which control blastula nuclei have given predominantly normal development. These experiments should provide a clearer indication of trends that have already been seen in Text-fig. 1, but which were then obscured by poor egg quality. In Text-fig. 2

the point at which each survival curve starts shows the percentage of total transplantations which became regular late blastulae.



TEXT-FIG. 2. Survival curves for *selected* experiments only (figures from Table 2). Transplant-embryo survival is expressed as a percentage of total transplantations for each donor age group.

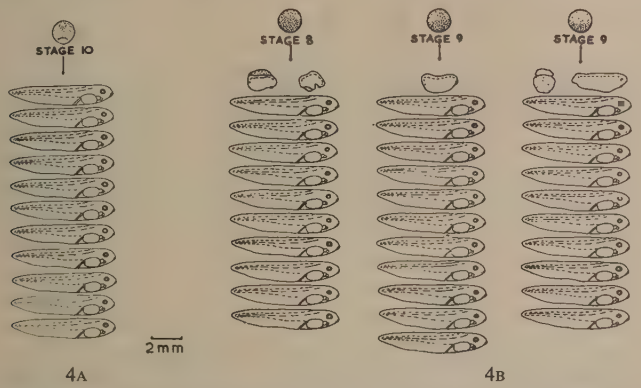


TEXT-FIG. 3. Survival curves for *selected* experiments only (figures from Table 2). Transplant-embryo survival is expressed as a percentage of the number of regular blastula transplant-embryos obtained from each donor age group.

In Text-fig. 3 transplant-embryo survival is shown as a proportion of late blastula transplant-embryos and not as a proportion of total transplantations. This method of presentation avoids the consequences of technical variation in the degree of donor-cell distortion, but only shows transplant-embryo survival beyond the late blastula stage. The survival curves in Text-fig. 3 are therefore believed to be a good representation of the innate qualities of different donor nuclei, since the effects of other factors which might affect these curves have been removed. Some of the 'selected' experiments have been shown in diagrammatic form (Text-figs. 4–9) and will be discussed below with the survival curves.

First-transfer experiments

The most normal transplant-embryo development that has been obtained from blastula nuclei is shown in Text-figs. 4B, 6, and 9. In these experiments nuclei from eight different blastulae have been tested. In the second series of Text-fig. 6 all transplant-embryos became normal tadpoles; in the other seven

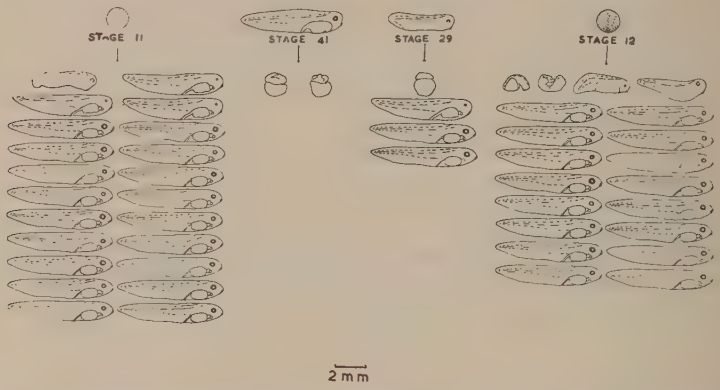


TEXT-FIG. 4. The development of transplant-embryos derived from the endoderm nuclei of an early gastrula (Text-fig. 4A), and from the nuclei of three late blastulae (Text-fig. 4B). Recipient eggs from the same frog were used for all three series in Text-fig. 4B, which have given very similar results. The total number of transplantations carried out for Text-fig. 4A was 14, and for each series of Text-fig. 4B, about 30.

In Text-figs. 4–9, only those transplant-embryos which survived as regular late blastulae are shown; transplanted eggs which cleaved irregularly have therefore been omitted. Further, the series are arranged according to the order in which they were done; that is, the series on the left was made first, and that on the right last.

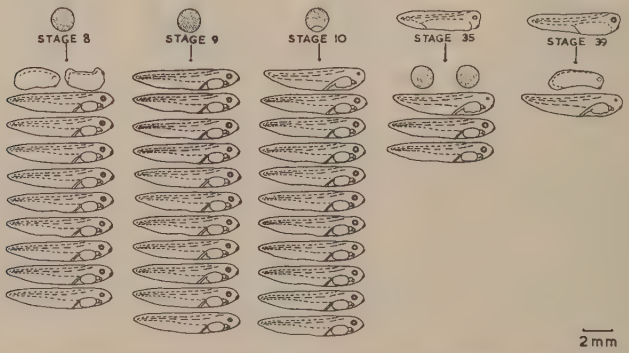
series most embryos were normal, but in each case a few developed abnormally. The cause of these abnormalities is not clear. It may be that the quality of *all* eggs is rarely good enough to withstand transplantation; it is often found in this laboratory that up to 10 per cent. of naturally fertilized eggs, obtained after hormone injection, develop abnormally; these abnormalities must presumably be attributed to poor egg quality. The small percentage of abnormal development obtained from blastula nuclei certainly does not justify the conclusion that

some of these nuclei are differentiated, or lack the potentiality for normal development. The present transplantation experiments are therefore believed to be entirely consistent with the view that blastula nuclei are undifferentiated. The survival curve for blastula nuclei in Text-fig. 3 will be used for comparison with the other survival curves for nuclei of later donor stages.



TEXT-FIG. 5. Transplant-embryos derived from nuclei of a mid-gastrula, a swimming tadpole, a heart-beat tadpole, and a late gastrula. About 40 transplantations were made for each series, and recipient eggs from the same frog were used throughout.

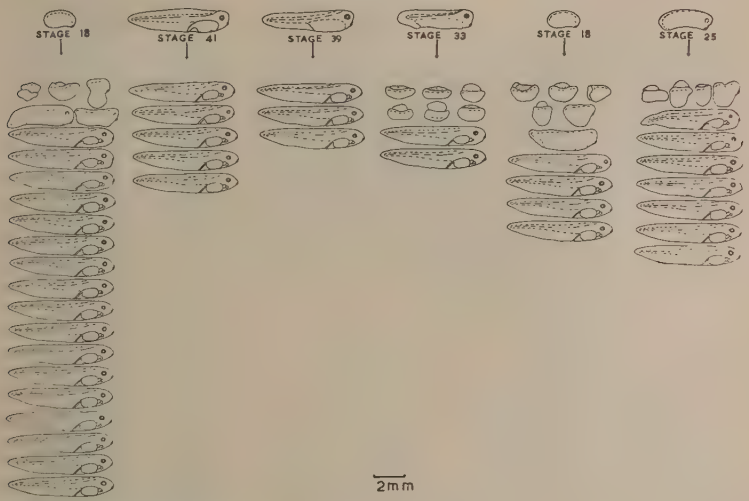
In this and all following diagrams, microcephalic and oedematous tadpoles are represented diagrammatically with a small eye and heart oedema in contrast to normal tadpoles (with a large eye). One microcephalic and two normal tadpoles are shown under the stage 29 donor.



TEXT-FIG. 6. Transplant-embryos from a mid-late blastula, a late blastula, an early gastrula, a hatching tadpole, and a swimming tadpole, all from recipient eggs of the same frog. Approximately 30 transplantations per series.

Gastrula nuclei have given the results shown in Text-figs. 4A, 5, 6, and 9. In the experiment depicted in Text-fig. 4A, 14 nuclei were transplanted; 3 eggs failed to cleave at all, but the remaining 11 cleaved regularly and all formed normal tadpoles which, had the experiment been continued, would almost

certainly have metamorphosed. Since the results of this experiment did not contain any abnormally cleaved eggs it constitutes particularly clear evidence that the endoderm nuclei from early gastrulae are totipotent and undifferentiated. In the other series with gastrula nuclei a few transplant-embryos are abnormal, though no more than with blastula nuclei. The survival curve for gastrula



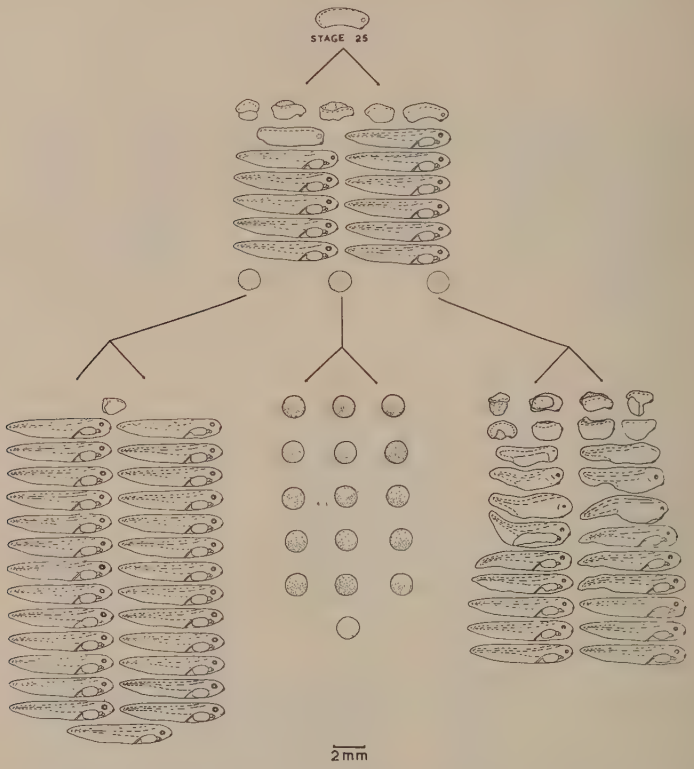
TEXT-FIG. 7. Transplant-embryos from a neural-fold stage, a stage 41 swimming tadpole, a stage 39 swimming tadpole, a heart-beat tadpole, another neural-fold stage, and a muscular-response stage; all into the eggs of one frog, and about 35 transplantations per series.

nuclei in Text-fig. 3 is within 5 per cent. of that for blastula donors at each point, a difference which is certainly not significant. There is no indication of any decrease in nuclear potentialities during gastrulation, since late blastulae and late gastrulae give similar results (compare the second and fifth series of Text-fig. 9).

It is worth drawing attention here to the consistent results that have been obtained when nuclei from several different blastula and gastrula donors are transplanted into the recipient eggs of one frog, as in Text-figs. 4B, 5, 6, and 9. The proportion of total transplantations which become late blastulae is very similar in these experiments, as shown by the number of embryos under each donor; also the proportion of late blastulae which become normal tadpoles is about the same in each case. The similarity in results is most marked among series in which recipient eggs of the same frog were used, that is, among all those shown in one figure. These consistent results would be expected from totipotent, undifferentiated nuclei.

Nuclei from older donor embryos appear to become progressively limited in their development potentiality. This differentiation is reflected not only in the proportion of total transplantations which form regular late blastulae, but

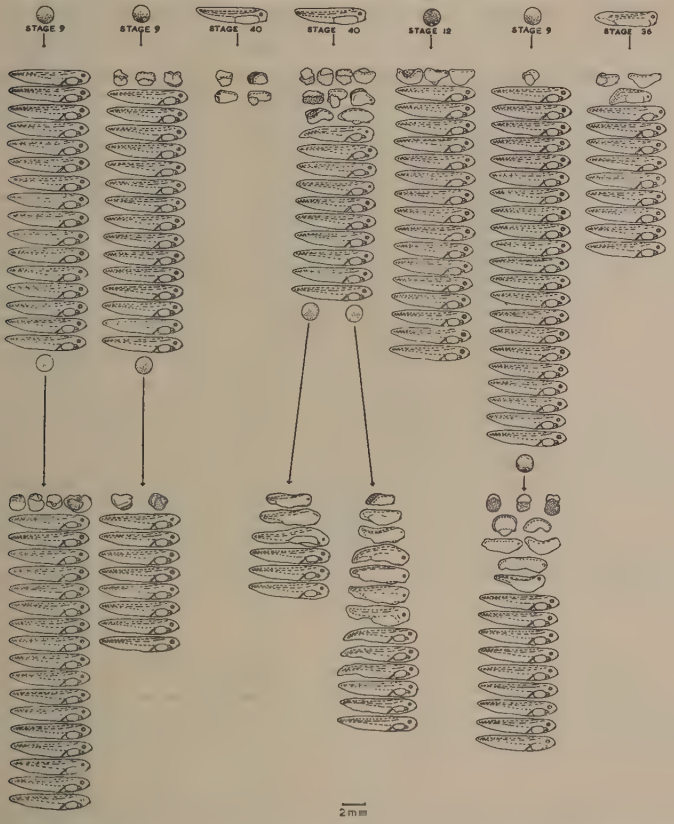
also in the proportion of late blastulae which develop normally. The point at which survival curves commence in Text-fig. 2 becomes lower with increasing age of the donor stage; this illustrates the decline in the proportion of late blastulae formed from older donor nuclei. It was pointed out above that the



TEXT-FIG. 8. Twenty transplant-embryos were obtained from endoderm nuclei of an original muscular-response stage tadpole. When these embryos had become late blastulae, three were used to provide nuclei for serial transplantations. The three resulting clones differ strongly from each other, but the transplant-embryos within each clone are very similar. About 50 transplantations were made for each of the four series. The serial clones (but not the first transfers) were made with the eggs of one frog.

technique may cause up to 20 per cent. of variation in the proportion of blastula transplant-embryos. Such variation in technique is probably responsible for the fact that a greater proportion of blastula transplant-embryos was obtained from neurula and gastrula donors than from earlier stages. In donors later than the heart-beat stage there is a pronounced reduction in the numbers of blastula transplant-embryos. This difference is too large to be accounted for by technical variation, and must therefore represent a real difference in nuclear potentialities. In most experiments the proportion of blastulae formed decreases

progressively from the heart-beat stage with increasing age of the donor; this typical condition is seen in Text-figs. 6 and 7, and particularly clearly in Text-fig. 5. However, this trend is not always consistent. It may happen that nuclei



TEXT-FIG. 9. First transfers were made from two late blastulae, two swimming tadpoles, a late gastrula, a late blastula, and a hatching tadpole. There is a marked difference in the results from the two pre-feeding tadpoles, in spite of consistently good quality recipient eggs (all from one frog) as shown by the three late blastula series. Five serial clones were made into the eggs of a second frog, using late blastula transplant-embryos as donors. It appears that the eggs of this second frog deteriorated towards the end of the experiment, since the proportion of abnormal transplant-embryos increases irrespective of donor age. About 50 transplantations were made for each series, both among the first transfers as well as in serial clones. The serial transplantations, like the first transfers, are arranged according to the order in which they were done, though the serial clones were of course not commenced until all first transfers had been completed.

from two donor embryos of exactly the same age, when transplanted into eggs of the same frog, will give entirely different results; this can be seen by comparing the 1st and 5th series of Text-fig. 7, and particularly clearly from a comparison of the 3rd and 4th series of Text-fig. 9. One may conclude from these results that

the ability of endoderm nuclei to form regular blastulae after transplantation becomes progressively reduced with increasing age of donor embryos beyond the heart-beat stage; on the other hand, the rate at which this capacity is lost is not always *closely* correlated with the developmental stage of the donor embryo.

The second respect in which endoderm nuclei appear to become differentiated concerns their decreasing capacity for giving normal transplant-embryo development. Blastula transplant-embryos derived from older donors are much less likely to become late gastrulae and normal tadpoles than transplant-embryos from younger donors (Text-fig. 3). This loss of capacity begins in nuclei from neurula donors. From this stage onwards the developmental potentiality of nuclei becomes increasingly restricted right up to the swimming tadpole stage, which is the latest from which nuclei have been transplanted. Even at this advanced stage, transplant-embryos from some nuclei have developed into normal tadpoles, though the proportion of such undifferentiated nuclei is very small.

As before, there is great variation in the extent to which this loss of capacity has proceeded in donor embryos of the same age. In Text-fig. 5 nuclei from a swimming tadpole have given wholly abnormal development, but in Text-fig. 7 two swimming tadpoles have given mostly normal development; the proportion of abnormalities is markedly different in the two swimming tadpoles of Text-fig. 9. Thus, though there is a clear tendency for more abnormal transplant-embryo development to be obtained from the nuclei of later donor stages, there is no *exact* correlation between the age of the donor embryo and the developmental potentialities of its endoderm nuclei.

Serial-transfer experiments

Serial nuclear transfers involve exactly the same technique as first-transfer experiments. The only difference is that the donor embryo is not derived from a normally fertilized egg, but is itself a transplant-embryo obtained by nuclear transplantation (King & Briggs, 1956). Reasons have been given above for believing that *Xenopus* endoderm nuclei do not differentiate until after the late gastrula stage; thus, if a transplant-embryo is used for serial transplantation before it has developed beyond a blastula or gastrula, all its endoderm nuclei should have the same potentiality as each other. If a blastula transplant-embryo derived from a normal nucleus is used as a donor, serial transplantation of its nuclei should yield a high proportion of normal development. On the other hand, if the original nucleus was differentiated so that it would have developed abnormally, then serial transplantation from it should (if the abnormality is inherited) provide many embryos (a clone) all suffering from the same kind of abnormality.

Two points can be investigated by serial transplantation of nuclei from transplant-embryos which have not developed beyond the blastula or gastrula stage. First, such experiments show whether the lack of potentiality of differentiated

nuclei is inherited or not, and, secondly, whether the differentiation of nuclei is reversible.

The extent to which nuclear changes are inherited is apparent from a comparison of one clone with another. If they are consistently different from each other this can only be attributed to heritable differences between the original donor nuclei. In Text-fig. 8 nuclei were taken from an original muscular response tadpole, and three transplant-embryos were used at the blastula stage to provide donor nuclei for serial clones. In the left-hand clone the great majority of embryos were normal; the proportion of abnormal embryos is very small and not more than is obtained with first-transfer blastula nuclei. This clone therefore shows that the original donor nucleus was undifferentiated. In the middle clone every transplant-embryo was arrested as a late blastula. The third clone on the right contains a range of abnormalities, but none of the tadpoles shown was quite normal, all being microcephalic and oedematous. The variation within this clone is discussed below. The three clones in this experiment are consistently different from each other, and so demonstrate clearly that the abnormalities in the first-transfer series are heritable.

In this experiment three blastula transplant-embryos were used to provide serial donor nuclei. It was not, of course, known how these three blastulae would have developed if they had been allowed to differentiate further. However, an indication of the way they might have developed can be derived from the development of the other 17 first-transfer embryos. The three serial donor embryos were chosen at random from 20 blastula transplant-embryos, and would therefore be expected to have developed in the same kind of way as did the 17 which were left. The potentialities of the 3 serial donors turned out to be consistent with the 17 first-transfer embryos. This result suggests that all the abnormalities present in the first-transfer group are heritable in the way that the abnormalities of the three embryos used for serial transplantation have been found to be.

The second kind of information which serial transplantation can give concerns the reversibility of nuclear differentiation. This is shown by the variation within a clone as opposed to the variation between clones. Clones from original blastula donors are shown in Text-fig. 9. Since the great majority of embryos in the first transfer series were normal, nuclei from embryos selected at random for serial donors would also be expected to be totipotent in the majority of instances. The first and second clones of Text-fig. 9 are predominantly normal; the clone from the 6th series contains more abnormal embryos than would be expected, but this series was carried out on the last eggs laid in one frog's ovulation, and some of these may have been immature. The first two clones of Text-fig. 9 and the first of Text-fig. 8 show that totipotent nuclei have no obvious tendency to age or to become abnormal after continued transplantation. These series also confirm that technique and egg quality cannot explain the abnormalities found in other clones. There is very little variation in clones from undifferentiated nuclei,

and no variation in those with differentiated nuclei (2nd clone of Text-fig. 8); however, clones in which no embryos are quite normal (though some are nearly so) are very variable. This is seen in the third clone of Text-fig. 8, and in the two clones from the fourth series of Text-fig. 9; in both cases the original donor was advanced and would therefore have contained differentiated nuclei. This variation could be explained in two ways. It might be that the potentiality of the original donor was like that of the worst or best embryo in the clone derived from it, and that after serial transplantation some nuclei increase or decrease their potentialities; or it might be that embryos obtained from abnormal nuclei are much more sensitive to technique, &c., than embryos with normal nuclei. In connexion with the latter possibility, haploid embryos, which have abnormal nuclei, differentiate very variably (Gurdon 1960*a*); though this variation might be due to genetic differences from one haploid nucleus to another, it might also be due to the effect of various environmental factors on nuclei suffering from the same abnormality (haploidy). Sufficient experiments have not yet been carried out to show whether variation in abnormal transplant-embryo clones represents a change in nuclear potentiality or not. Until other possibilities have been excluded, this limited variation cannot be regarded as evidence that nuclear differentiation is reversible.

CONCLUSIONS CONCERNING THE POTENTIALITIES OF ENDODERM NUCLEI IN *XENOPUS*

Nuclear differentiation in relation to the developmental stages of donor embryos

The nuclei of differentiating endoderm cells become progressively limited in their developmental capacity. This differentiation is evident not only from the proportion of transplantations which result in late blastulae, but also from the proportion of blastula transplant-embryos which develop normally. The proportion of late blastulae obtained from the nuclei of embryos up to the muscular response stage does not vary, but becomes progressively reduced from nuclei of heart-beat and later stages. Although variation in technique can affect the proportion of late blastulae, it cannot account for the great difference in this respect between nuclei from young and advanced donor embryos. The majority of transplant-embryos derived from blastula and gastrula nuclei develop normally as far as swimming tadpoles; however, the more advanced the donor, the more transplant-embryos develop abnormally, and the more severe are the abnormalities from which they suffer.

These results indicate that the endoderm nuclei of *Xenopus* only *begin* to become differentiated in embryos older than late gastrulae. From the neurula stage onwards there is a progressive increase in the proportion of differentiated nuclei, but even in swimming tadpoles there remain a few endoderm nuclei with entirely unrestricted developmental potentialities.

These conclusions have been based on the results of selected experiments. It was, however, pointed out (p. 509) that the same conclusions can be drawn,

though less obviously, from *all* experiments. Fischberg, Gurdon, & Elsdale (1958) reached similar general conclusions from some earlier experiments with *Xenopus*.

Limited application of results

Attention must now be drawn to certain limitations which apply to these results. Only endoderm nuclei have been investigated, but comparable changes will probably be found to take place in the nuclei of other differentiating tissues (Briggs & King, 1957).

The potentialities of nuclei have not been tested for *all* developmental stages up to that in which the endoderm is differentiated. No transplantations have been made from nuclei of earlier stages than a mid-blastulae, but there is no reason to believe that such nuclei would give more normal development than was obtained from late blastula and gastrula nuclei. The latest donor stage from which nuclei were transplanted was a swimming tadpole in which the gut had just formed two right-angled bends. In order to transplant from later stages, other substances than Versene would be required for cell disaggregation, and it is not certain that these would be harmless. The potentialities of endoderm nuclei have therefore been tested only between Nieuwkoop's stages 8 and 41, but some gut cells remain undifferentiated until stage 46, several hours later.

The development of transplant-embryos has only been followed up to the formation of normal tadpoles. Transplanted nuclei have therefore been tested only for their ability to bring about embryonic development up the tadpole stage. Transplant-embryos which become normal tadpoles can be reared into sexually mature frogs (Gurdon, Elsdale, & Fischberg, 1958); the study of these frogs gives additional information about the nuclei from which they were derived, and will be dealt with elsewhere (Gurdon, in preparation).

The part of a nucleus in which differentiation takes place

The changes undergone by differentiating endoderm nuclei were described above as 'innate nuclear qualities'. This term refers to the conclusions based on transplant-embryo development when all effects of the transplantation technique and egg quality have been removed from the results. The evidence for nuclear differentiation is of two kinds, concerning first the proportion of total transplants which form late blastulae, and, secondly, the proportion of late blastulae which develop normally. Nuclei from late donor stages such as hatching tadpoles undergo mitosis rather seldom (see below), but after transplantation are required suddenly to enter a phase of frequent mitoses (cleavage). Those nuclei which are differentiated so as not to be able to do this—that is, those which cannot form regular late blastulae—have not been used for serial transplantation. Thus nothing is known about the heritability or eventual reversibility of this kind of nuclear differentiation, which seems unlikely to be connected with the chromosomal parts of the nucleus.

The other kind of nuclear differentiation which affects the ability of late blastula transplant-embryos to develop normally has been shown by serial transplantation to be both heritable and, to a large extent at least, irreversible. These two characteristics are associated with gene-controlled factors. Moreover, it is shown below that there is no correlation between these nuclear changes and certain general, reversible nuclear qualities, such as size and phase of mitosis. Most nuclear components other than the chromosomes are changed at each mitosis; these nuclear changes, which are heritable, cannot therefore be associated with the nuclear membrane, nuclear sap, or nucleolus. Thus there are strong reasons for believing, as in *Rana* (King & Briggs, 1956), that the changes in endoderm nuclei demonstrated by the development of late blastula transplant-embryos have taken place in the chromosomes.

The smallest endoderm nuclei to have been transplanted (stage 41) are at least as large and as well protected by cytoplasm as nuclei from the animal hemisphere of a blastula; but, as animal and vegetal blastula nuclei give equally good results, nuclear changes cannot be associated with the size of nuclei (Gurdon, 1960*b*). The proportions of endoderm nuclei 'in mitosis' (nuclear membrane disappeared) has been counted in embryos at different stages of development, reared at 21° C. One hundred endoderm nuclei of the kind used as donors for nuclear transplantation were counted for each donor stage. The proportion of nuclei in mitosis was 17 per cent. in a blastula, 7 per cent. in a late gastrula, 5 per cent. in a neural-folds embryo, 2 per cent. in a heart-beat embryo, and 1 per cent. in a stage 41 swimming tadpole. These figures give only a rough indication of the frequency with which nuclei in mitosis were used for transplantation; they show, however, that the transplantation of mitotic nuclei, whatever effect this may have, is too infrequent to affect the evidence for nuclear differentiation.

The significance of nuclear changes

There is no obvious correlation between nuclear differentiation and the morphogenetic development of tissues. This is shown below (p. 521) by comparison of nuclear differentiation in *Rana* and *Xenopus*. Very little seems to be known about the state of determination of endoderm tissue in *Xenopus*. Though the endoderm of Amphibia is described as 'regionally determined histologically some time before gastrulation' by Kemp (1951), Okada (1957) has shown that pharyngeal endoderm from a neurula can form many different endoderm structures. As it is not known what other tissues endoderm can form when grafted into various regions of an embryo, it does not seem possible to compare tissue determination and nuclear differentiation. It seems possible, however, that nuclear differentiation may be connected with cell differentiation. In *Xenopus* the nuclei used for transplantation have been taken from the most undifferentiated endoderm cells of each donor stage. As the gut becomes formed, many of the peripheral endoderm cells become differentiated—that is, they lose their more or less spherical shape, and their nuclei often become elongated;

these cells also become very strongly connected to each other, and it is for this reason that nuclei have not been transplanted from fully differentiated cells. What has in effect been done in these experiments is to transplant nuclei from undifferentiated endoderm cells, and to show that the nearer the time of cellular differentiation, the more nuclei become differentiated. These results are consistent with the considerable variation that has been found in the differentiation of nuclei from embryos of late donor stages (p. 516). Some donor cells were probably nearer this differentiated state than others. It is therefore suggested that nuclear differentiation may play a significant part in the processes which take place when individual cells attain their final functional state. This hypothesis can only be tested when a method is found by which differentiated cells can be harmlessly dissociated.

COMPARISON OF NUCLEAR DIFFERENTIATION IN *XENOPUS LAEVIS* AND *RANA PIPIENS*

Briggs & King (1957, 1960), using *R. pipiens*, have transplanted endoderm nuclei from donors of various ages between late blastulae and tail-bud embryos. The survival of transplant-embryos derived from *R. pipiens* nuclei is shown in

TABLE 3

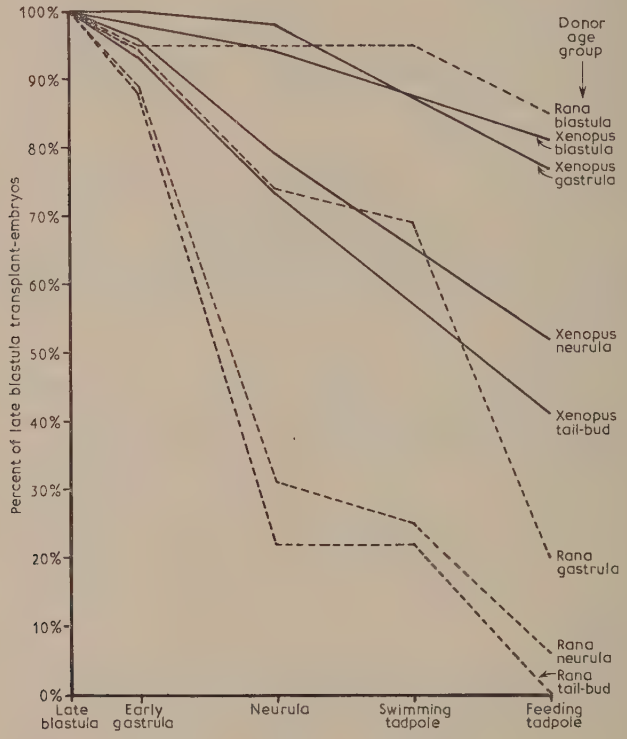
A comparison of nuclear differentiation in Rana *and* Xenopus

The percentages are expressed in terms of the number of late blastula transplant-embryos for each donor stage. The figures for *Rana* have been taken from Briggs & King (1957), and for *Xenopus* from Table 2 above.

Donor stage	*Species*	*Total transplantations*	*Stages in transplant-embryo development*				
			Late blastulae	*Early gastrulae*	*Neurulae*	*Tail-buds*	*Normal tadpoles*
Late blastula or early gastrula animal cells, (undifferentiated nuclei)	*Rana*	92	39 100%	37 95%	37 95%	37 95%	33 85%
	Xenopus	327	100%	98%	94%		81%
Late gastrula endoderm	*Rana*	155	79 100%	75 95%	58 74%	54 69%	16 20%
	Xenopus	502	100%	100%	98%		77%
Neurula endoderm	*Rana*	98	16 100%	14 88%	5 31%	4 25%	1 6%
	Xenopus	140	100%	96%	79%		52%
Tail-bud endoderm	*Rana*	130	9 100%	8 89%	2 22%	2 22%	0 0%
	Xenopus	152	100%	94%	75%		41%

Table 3 and has been expressed as a percentage of late blastula transplant-embryos for each donor stage; these figures are therefore comparable to those taken from Table 2 for *Xenopus*. The survival of transplant-embryos in *Rana* and *Xenopus* is compared in Table 3 and Text-fig. 10.

Transplant-embryos derived from blastula nuclei of *Rana* are mostly normal; there is, however, a sharp decline in the developmental potentialities of endoderm nuclei from gastrulae and later stages. Thus in *Rana* the differentiation of endoderm nuclei starts at the beginning of gastrulation and continues from that stage onwards; by the tail-bud stage all endoderm nuclei are differentiated, since none has given rise to normal transplant-embryo development (Briggs & King, 1957).



TEXT-FIG. 10. Survival curves for endoderm nuclei of *Rana* (dotted lines) and *Xenopus* (solid lines); the numbers have been taken from Table 3. Each curve shows the survival in terms of the late blastula transplant-embryos of one donor stage.

In *Xenopus*, on the other hand, the differentiation of endoderm nuclei does not begin until the neurula stage, and then only takes place very gradually from that stage onwards; even in swimming tadpoles some endoderm nuclei remain undifferentiated. In both *Rana* and *Xenopus* a similar proportion of normal transplant-embryos are obtained from undifferentiated blastula nuclei, but in other respects the rate and time of onset of endodermal nuclear differentiation are very different for the two species.

The possibility that transplant-embryo survival is affected by differences in

the technique for the two species seems improbable (Elsdale, Gurdon, & Fischberg, 1960). The only significant difference in technique concerns the method of enucleation of recipient eggs. In *Rana* the egg nucleus is removed, while in *Xenopus* it is inactivated by irradiation and allowed to degenerate in the egg cytoplasm (Gurdon, 1960*a*). In *Xenopus* there is no reason to suppose that the dying egg nucleus has any effect on either the injected nucleus or the egg cytoplasm, but this question cannot be finally settled until it is possible to enucleate the eggs of both species by the same method.

The *Xenopus* transplantations described in Text-fig. 10 are selected results, while the results for *Rana* are the average of all experiments with endoderm nuclei. The use of selected results for *Xenopus* does not, however, affect the conclusions, since a similar comparison with *Rana* is obtained if the total results for *Xenopus* (Table 1) are used instead of selected results. It appears that Briggs & King, with *Rana*, have not found much variation corresponding to the eggs of individual frogs. They have used frogs which have been taken from their natural conditions, and this might account for the eggs of *Rana* being more consistent in quality than those of *Xenopus*. It could, on the other hand, be that the much greater size of *Rana* eggs renders them less susceptible to damage by experimental manipulation, so that all *Rana* eggs are resistant to the technique, while this is only true of *some* eggs in the case of *Xenopus*. Whichever of these possibilities is correct, it cannot invalidate the very marked difference in nuclear differentiation between the two species.

Both *Rana* and *Xenopus* pass through very similar stages of morphological differentiation. Cleavage, gastrulation, and elongation of the embryo are proportionately faster in *Xenopus*, but this is largely due to the higher temperature at which *Xenopus* develops. The rate of embryonic development does not appear to affect the rate of nuclear differentiation, since nuclei from *Xenopus* embryos which were reared at 18° C. and 26° C. gave a similar proportion of normal and abnormal transplant-embryos. These experiments lead to the conclusion that no consistent relationship exists between the rate of nuclear differentiation and that of morphological tissue differentiation. This confirms the conclusion (p. 520) that there is no exact correlation between the differentiation of a tissue and that of its cell nuclei.

SUMMARY

1. The developmental potentiality of embryonic endoderm nuclei in *X. laevis* is shown to change as the tissue to which they belong becomes differentiated. This change has been demonstrated by taking nuclei from endoderm tissue in different stages of differentiation, and transplanting them into enucleated unfertilized eggs; the development of the resulting transplant-embryos indicates the developmental capacity of their nuclei.

2. Since the proportion of total transplantations which become late blastulae is affected by the technique, the main conclusions have been drawn from the

further development of late blastula transplant-embryos. The quality of recipient eggs, which is always variable, may also affect transplant-embryo development, and conclusions have therefore been mainly derived from selected experiments in which control donor nuclei have shown that egg quality was good. Reasons have been given for believing that non-hereditary nuclear qualities, such as their size and stage in mitosis, do not affect the conclusions drawn from transplant-embryo development; their development is therefore solely dependent on the specific genetic qualities of the donor nuclei used.

3. No decline in the developmental potentiality of endoderm nuclei was found in blastulae and gastrulae. However, the capacity of nuclei to form normal tadpoles decreased progressively from after gastrulation until the beginning of torsion in the gut of swimming tadpoles; at this late stage there was still a small proportion of undifferentiated nuclei from which normal tadpoles have been obtained. Thus nuclear differentiation affects an increasing proportion of nuclei to an increasing extent, as the endoderm becomes differentiated. Serial transplantation has shown that the changes involved in nuclear differentiation are heritable and, at least to a large extent, irreversible.

4. These results are compared with those of Briggs & King who transplanted nuclei from endoderm cells of *R. pipiens*. In both *Xenopus* and *Rana*, nuclei are undifferentiated at the late blastula stage, but after this the rate and time of onset of nuclear differentiation is very different for the endoderm of the two species. These differences show that there is no exact correlation between nuclear differentiation and tissue differentiation. It is suggested that nuclear differentiation may be concerned in the processes which take place when individual cells become differentiated into their final functional state.

RÉSUMÉ

La capacité de développement de noyaux prélevés sur les cellules endodermiques en voie de différenciation de Xenopus laevis

1. La capacité de développement des noyaux endodermiques de l'embryon de *Xenopus laevis* se modifie quand le tissu auquel ils appartiennent se différencie. Ce changement est démontré par le prélèvement des noyaux du tissu endodermique à différents stades de la différenciation et par leur transplantation dans des œufs fécondés énucléés; le développement des embryons-transplants qui en résultent manifeste la capacité de développement de leurs noyaux.

2. Puisque la proportion de transplantations totales qui donnent des blastulas âgées est affectée par cette technique, les conclusions principales ont été tirées du développement ultérieur des blastulas âgées issues des embryons-transplants. La qualité des œufs servant d'hôtes, qui est toujours variable, peut aussi affecter le développement de l'embryon-transplant, et des conclusions ont donc été tirées principalement d'expériences sélectionnées, dans lesquelles les donneurs de noyaux témoins ont montré que la qualité de l'œuf était bonne. On donne des

raisons pour lesquelles il y a lieu de penser que des propriétés non héréditaires des noyaux, telles que leur taille et leur stade mitotique, ne modifient pas les conclusions que l'on peut tirer du développement des embryons-transplants. Leur développement ne dépend donc que des propriétés génétiques spécifiques des noyaux donneurs utilisés.

3. Il n'a été trouvé aucune diminution du pouvoir de développement des noyaux endodermiques dans les blastulas et les gastrulas. Cependant, le pouvoir qu'ont les noyaux de former des têtards normaux décroît progressivement depuis la gastrulation jusqu'au début de la torsion de l'intestin chez les têtards nageants; à ce dernier stade, il reste encore une petite proportion de noyaux indifférenciés, à partir desquels on a obtenu des têtards normaux. Ainsi la différenciation nucléaire atteint un nombre croissant de noyaux, quand l'endoderme vient à se différencier. Des transplantations sériées ont montré que les changements impliqués dans la différenciation nucléaire sont transmissibles et, au moins pour une large part, irréversibles.

4. Ces résultats sont comparés à ceux de Briggs & King, qui ont transplanté des noyaux de cellules endodermiques de *Rana pipiens*. Chez *Xenopus* comme chez *Rana*, les noyaux sont indifférenciés au dernier stade de la blastula, mais après cela la vitesse et l'installation de la différenciation nucléaire sont très différentes pour l'endoderme des deux espèces. Ces différences montrent qu'il n'y a pas de lien étroit entre la différenciation nucléaire et la différenciation tissulaire. On suggère que la différenciation nucléaire jouerait un rôle dans les processus qui se placent au moment où les cellules individuelles acquièrent leur différenciation finale et fonctionnelle.

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REFERENCES

Barth, L. G., & Barth, L. J. (1959). Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. exp. Morph.* **7**, 210–22.

Briggs, R., & King, T. J. (1953). Factors affecting the transplantability of nuclei of frog embryonic cells. *J. exp. Zool.* **122**, 485–505.

—— —— (1957). Changes in the nuclei of differentiating endoderm cells as revealed by nuclear transplantation. *J. Morph.* **100**, 269–312.

—— —— (1960). Nuclear transplantation studies on the early gastrula (*Rana pipiens*). I. Nuclei of presumptive endoderm. *Devel. Biol.* **2**, 252–70.

ELSDALE, T. R., FISCHBERG, M., & SMITH, S. (1958). A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp. Cell Res.* **14**, 642–3.

—— GURDON, J. B., & FISCHBERG, M. (1960). A description of the technique for nuclear transplantation in *Xenopus laevis*. *J. Embryol. exp. Morph.* **8**, 437–44.

FISCHBERG, M., GURDON, J. B., & ELSDALE, T. R. (1958). Nuclear transfer in Amphibia and the problem of the potentialities of the nuclei of differentiating tissues. *Exp. Cell Res. Suppl.* **6**, 161–78.

GURDON, J. B. (1959). Tetraploid frogs. *J. exp. Zool.* **141**, 519–44.

—— (1960*a*). The effects of ultraviolet irradiation on the uncleaved eggs of *Xenopus laevis*. *Quart. J. micr. Sci.* **101**, 299–312.

—— (1960*b*). Factors responsible for the abnormal development of embryos derived from transplanted nuclei in *Xenopus laevis*. *J. Embryol. exp. Morph.* **8**, 327–40.

—— (in preparation). The reproductive capacity and other characters of adult *Xenopus laevis* derived from embryonic endoderm nuclei.

—— ELSDALE, T. R., & FISCHBERG, M. (1958). Sexually mature individuals of *Xenopus laevis* derived from the transplantation of single somatic nuclei. *Nature*, **182**, 64–65.

KEMP, N. E. (1951). Development of intestinal coiling of Anuran larvae. *J. exp. Zool.* **116**, 259–87.

KING, T. J., & BRIGGS, R. (1955). Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. *Proc. nat. Acad. Sci. Wash.* **41**, 321–5.

—— —— (1956). Serial transplantation of embryonic nuclei. *Cold. Spr. Harb. Symp. quant. Biol.* **21**, 271–90.

NIEUWKOOP, P. D., & FABER, J. (1956). *Normal Table of* Xenopus laevis (*Daudin*). Amsterdam: North-Holland Publishing Company.

OKADA, T. S. (1957). The pluripotency of the pharyngeal primordium in Urodelan neurulae. *J. Embryol. exp. Morph.* **5**, 438–48.

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Growth and Ascorbic Acid Content of the Chick Embryo

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ASCORBIC acid (ASA) is actively synthesized by germinating plant seeds (see Mapson, 1953), and by the embryos of various animal species (refs. in Needham, 1942).

Hauge & Garrick (quoted by Needham, 1931) found no ASA in the unincubated hen's egg. This was confirmed by Ray (1934), who showed that the vitamin C content of the chick embryo increases gradually after incubation of the egg. Since the egg is a closed system, it follows that the chick embryo can synthesize its own ASA and that the ASA content of the embryo at any given stage must be the balance between synthesis and utilization.

It was, therefore, considered of interest to make daily weighings and ASA estimations throughout development with the more sensitive methods now available in order to examine the possible relations between embryonic weight and ASA content on the one hand, and between growth rate and ascorbic acid concentration on the other. ASA concentration was also measured on some organs of early and late embryos and of adult birds.

MATERIAL AND METHODS

Growth measurements

Daily weighings were made on Light Sussex chick embryos from the second day of incubation till hatching.

Special precautions were taken in weighing early embryos to avoid errors due to evaporation and to prevent oxidation or loss of ASA in solution. The area pellucida was freed from the yolk and transferred to a hollow-ground slide inside a moist chamber, where the embryos were quickly but thoroughly freed from all extra-embryonic material, without using saline. The embryos were kept chilled in a stoppered weighing bottle, and before the last specimen was collected the container was wiped dry and its temperature was allowed to equilibrate inside the balance case. As soon as the last embryo was dissected, the pooled specimens were weighed in a semi-automatic balance. Stainless steel

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instruments were used to prevent catalytic oxidation of ASA by traces of iron or copper.

The dry weight was calculated from the wet weight according to Murray's figures of percentage water content, quoted by Needham (1931). It may be noted that the embryo becomes dryer as it develops, but dehydration is not linear; the fastest rate is from the 11th to the 15th day. The average daily variation in water content is less than 1 per cent. of the embryo's weight, and therefore the error involved in calculating the water content should be negligible, even allowing for some difference between the material used here and that of Murray.

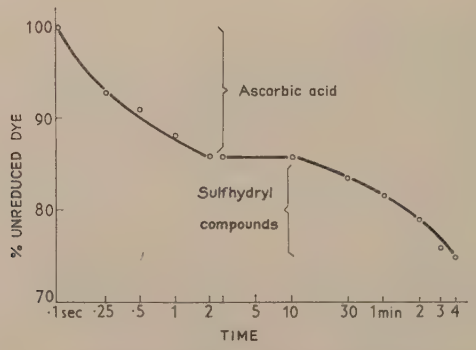
Several samples were weighed and analysed for each day of development. At the early stages, when the embryos were small, several specimens were pooled in each sample to decrease the experimental error.

Vitamin C estimations

The vitamin C estimations were made simultaneously with the growth measurements on the same material. Extraction of the vitamin with 5 per cent. metaphosphoric acid was done immediately after weighing the specimens. For small samples—early embryos or organs—the acid was added directly into the weighing bottle. Otherwise the specimens were ground with acid-washed sand and HPO_3 in a mortar.

Two methods were used for the vitamin C estimations: visual titration with 2,6-dichlorophenolindophenol according to the technique of Birch, Harris, & Ray (1933) and osazone formation with 2,4-dinitrophenylhydrazine (Roe & Kuether, 1943; Roe & Oesterling, 1944) as modified by Bolin & Book (1947). All solutions were made up with copper-free glass-distilled water and the dye solution was regularly standardized against pure crystalline ASA.

For dye titration the filtered extracts were adjusted to contain approximately 50 γ of ASA per ml. Below this concentration the accuracy of the method declines steeply. This method was used only when enough filtrate could be obtained for making triplicate titrations.



TEXT-FIG. 1. Continuous flow filtration of ASA from chick embryos.

The specificity of the dichlorophenolindophenol method is satisfactory for animal tissues, where the few substances known to interfere within the same range of redox potentials at pH lower than 5, such as glutathione or cysteine, reduce the dye at a much slower rate than ASA, so that interference can be avoided by carrying out the titration in less than 30 seconds (Harris *et al.*, 1948). The specificity in chick embryonic material was checked by the 'continuous flow' method of Harris & Mapson (1947). Text-fig. 1 shows the amount of dye consumed during the first 2·5 seconds by a sample extract of chick embryos titrated in the flow apparatus at pH 4·5. This was equivalent to 69 γ of ASA/ml., exactly the same titre as that obtained by the visual titration technique in 30 seconds. The second part of the curve is similar to the calibration curves obtained with reduced glutathione or cysteine.

The dinitrophenylhydrazine method was used exclusively for all estimations in early embryos and individual organs when the sensitivity of the oxidation-reduction method was not sufficient, and also in later stages as a complement and a check to dye titrations. The osazone method is some fifty times more sensitive than the visual titration method and it has the further advantage that oxidized ASA also can be estimated. Reducing substances occurring in embryonic tissues, such as sulphydryl compounds, do not interfere, but glucuronic acid and some of the precursors of ASA may. Thus, with the possible exception of reductones that are not present in fresh animal tissues, there is no overlapping of interfering substances between this and the dye titration method, so that both could be used together for testing the specificity of ASA estimations. With freshly obtained embryonic tissues, the two methods agreed within 10 per cent.

Bolin & Book's modification of Roe's method was found to have several advantages. Firstly, less filtrate is required; secondly, the risk of loss of vitamin by adsorption on charcoal is eliminated; and thirdly, the colour remaining after adding a drop of dye to the solution gives a rough indication of its titre and shows whether further dilution is necessary. The technique was standardized with pure ASA (Roche) oxidized with bromine, according to Roe's original procedure. Almost perfect agreement was obtained between aliquots with bromine and with dichlorophenolindophenol. The colour obtained with dinitrophenylhydrazine was found to be stable for 48 hours at 4° C., so that the readings could be rechecked when necessary. A blank was obtained for every sample analysed, and reagent blanks were also run frequently. All samples were suitably diluted to fall within the linear range of the method, which was from 0·5 to 10 γ of ASA/ml.

RESULTS

Growth measurements

The results of daily weighings are shown in Table 1 and in Text-fig. 2. It may be noticed that the curves obtained for both wet and dry weight are sigmoid.

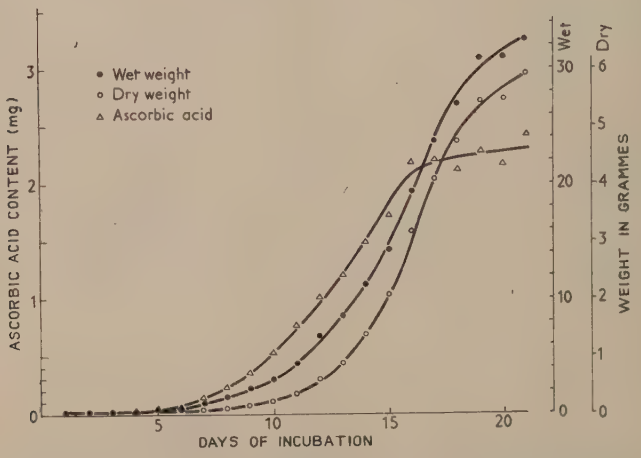
The coefficient of variation showed only small fluctuations along the curve, except for the 3rd day.

TABLE 1

Weight of chick embryos

Figures in brackets indicate weight with 'spare yolk' included (see text).

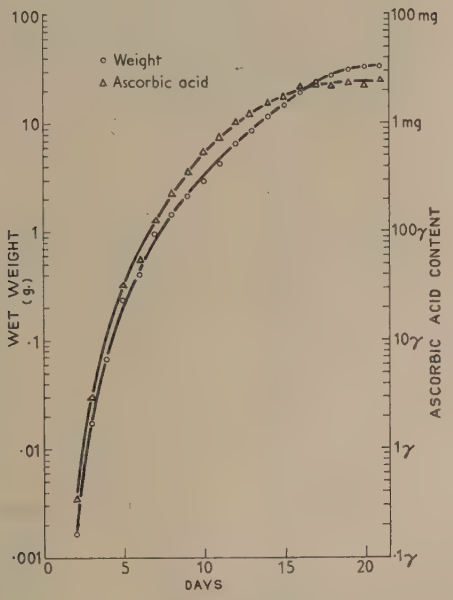
Days	*Number of embryos*	*Number of samples*	*Average wet weight per embryo (g.)*	*Coefficient of variation*	*Average dry weight per embryo (g.)*	*Growth rate (dw/dt)*	*Specific growth rate (1/w) (dw/dt)*
2	13	3	0·00162	0·002	0·000075	..	..
3	31	4	0·0169	2·516	0·0008	..	..
4	10	3	0·0665	0·605	0·0033	0·107	1·61
5	14	4	0·227	0·501	0·012	0·144	0·63
6	14	7	0·397	0·084	0·023	0·359	0·90
7	6	4	0·935	0·011	0·055	0·525	0·56
8	3	3	1·418	0·000	0·086	0·571	0·40
9	4	4	2·094	0·004	0·136	0·654	0·31
10	5	3	2·812	0·054	0·197	0·993	0·35
11	5	3	4·241	0·006	0·318	2·043	0·48
12	4	3	6·670	0·003	0·587	2·107	0·32
13	4	3	8·443	0·002	0·852	2·167	0·26
14	2	2	11·16	0·005	1·36	2·769	0·25
15	6	5	14·18	0·007	2·05	4·184	0·30
16	8	6	19·34	0·017	3·18	5·008	0·26
17	7	6	23·65	0·018	4·07	3·59	0·15
18	4	3	26·82	0·000	4·72	3·89	0·15
19	4	4	30·94	0·003	5·48	2·05	0·07
20	4	4	31·00 (39·51)	0·004	5·60	..	..
21	6	6	32·55 (45·20)	0·002	5·90	..	..



TEXT-FIG. 2. Wet and dry weight and ASA content of chick embryos.

As is well known, the yolk sac is incorporated into the abdominal cavity before hatching, usually after the 19th day of development. This causes a sudden increase in weight, and it is debatable whether the added mass contributed by the extra-embryonic structures should be computed as embryonic weight or whether it should be excised before weighing the embryo.

The incorporation of the yolk sac may be one of the reasons why some workers (see Needham, 1931) have missed the flattening of the curve at the end of development; their figures give the wrong impression that the growth-curve of the chick embryo is exponential, and not sigmoid.



TEXT-FIG. 3. Wet weight and ASA content per embryo (semi-log. plotting).

The weight-curve has a point of inflexion between the 15th and 17th days which is in close agreement with the findings of Lamson & Edmund, Hasselbalch, and Schmalhausen (see Needham, 1931). Consequently, the peak of the first derivative dw/dt, or velocity curve, is at the 16th day as shown in Table 1. (In this and all the following equations w stands for weight and t for time.)

The semi-log. plotting (Text-fig. 3) shows a hyperbola concave to the abscissa, emphasizing the fact that the specific growth rate is highest at the beginning and decreases continuously throughout development. The 'specific growth rate' $(1/w)\,(dw/dt)$ (Table 1) plotted against time approximates an inverted hyperbola which shows a deceleration with time.

An attempt to find a reasonably simple equation which would fit the data

approximately indicated that the first portion of the curve was fitted by a Gompertz equation while the middle and late parts were better fitted by a logistic curve.

Vitamin C estimations

The negative findings of earlier workers for the yolk and the white of unincubated eggs were confirmed in the present experiments by means of the more sensitive phenylhydrazine method. Even with this method it is not possible to rule out the presence of traces of reduced or oxidized vitamin smaller than 0·5 γ per ml. in the white or the yolk, but as the eggs weighed approximately 50 g. without the shell, they could not contain more than 25 γ at most before incubation, while the embryos were found to contain more than 30 γ at the 5th day of incubation, and nearly 2·5 mg. at hatching. One may conclude, therefore, that the chick embryo actively synthesizes ASA.

TABLE 2

Ascorbic acid content of chick embryos

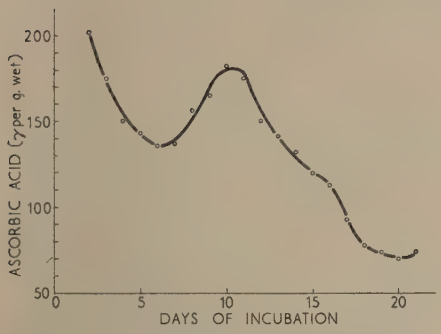
Days	*Average ASA content per embryo*	*Coefficient of variation*	*Average ASA content per g. wet weight*	*Coefficient of variation*	*Average ASA content per mg. dry weight*
2	0·33 γ	0·011	204 γ	0·069	4·0 γ
3	2·95	3·597	175	0·044	3·7
4	10	0·050	150	0·486	3·3
5	32	0·246	143	0·114	2·7
6	54	0·271	136	0·140	2·3
7	128	0·048	137	0·012	2·3
8	222	0·015	156	0·016	2·6
9	345	0·003	165	0·013	2·5
10	514	0·170	182	0·023	2·6
11	752	0·017	175	0·000	2·4
12	1,000	0·011	150	0·003	1·7
13	1·19 mg.	0·003	141	0·000	1·4
14	1·48	0·005	132	0·000	1·1
15	1·71	0·032	120	0·012	0·83
16	2·19	0·030	113	0·025	0·69
17	2·20	0·006	93	0·018	0·54
18	2·11	0·036	78	0·012	0·45
19	2·27	0·038	74	0·063	0·41
20	2·16	0·008	70	0·003	0·39
21	2·42	0·000	74	0·008	0·41

Vitamin C appears very early in the embryo. A positive reaction was obtained with Bolin & Book's procedure on 24-hour blastoderms, and at 48 hours the embryos already contained 0·3 γ on an average.

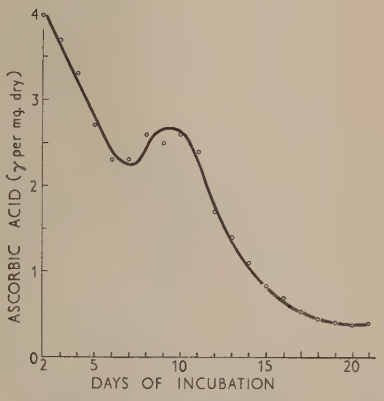
The results of daily estimations of ASA in whole embryos are shown in Table 2 and in Text-figs. 2 and 3. It may be noticed that there is a high correlation between the ASA content of the embryo and its weight up to the point of inflexion of the weight-curve (16th day), at which point the vitamin content

ceases to increase altogether. The change in ASA content per embryo showed a fairly good approximation to a logistic curve.

The relative increase in vitamin in respect to the increase in total embryonic mass was calculated with Huxley's (1932) formula $y = bx^k$, where b is a constant, y is the magnitude of the part, in this case vitamin C, x the magnitude of the whole, in this case embryonic weight, and k is the 'heterauxetic constant'. The double logarithmic plotting resulted in a series of straight lines, but instead of quoting different heterauxetic constants for different stages of development, it was preferred to find the nearest linear approximation by the least square method and to give a single k which can be compared with that of other compounds. The value of k thus obtained was 0·896.



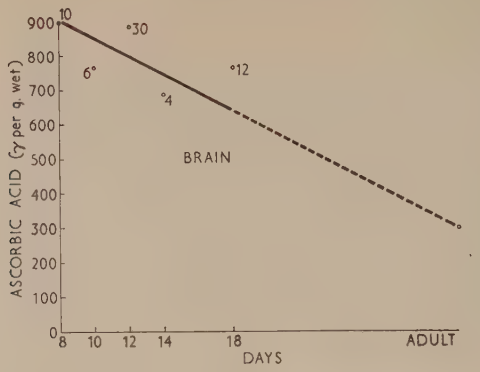
TEXT-FIG. 4. Concentration of ASA per gram of wet tissue.



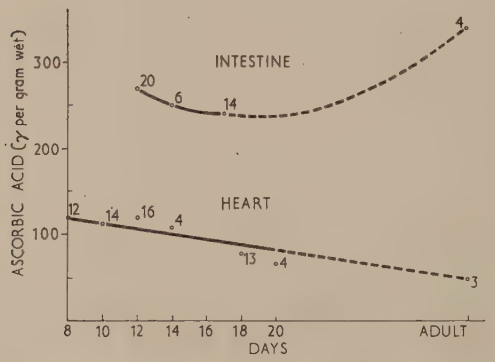
TEXT-FIG. 5. Concentration of ASA per gram of dry tissue.

The concentration of ASA per gram of wet tissue is shown in Text-fig. 4, and that per gram of dry tissue in Text-fig. 5. From the 2nd day of development until hatching the ASA concentration showed a decline from 204 γ to 74 γ per g. of wet embryo and from 4·0 to 0·41 γ per mg. dry embryo. For dry tissue, therefore, the decline is of the same order as that of the growth rate, i.e. to about 1/10 of the initial value. For wet tissue, on the other hand, the growth rate falls to about 1/8 while the ASA concentration declines to only about 1/3. Moreover, the curves of specific growth rate and of ASA concentration are not parallel. This discourages the suggestion of a simple correlation between the concentration of vitamin C and the rate of cell-division in the embryo.

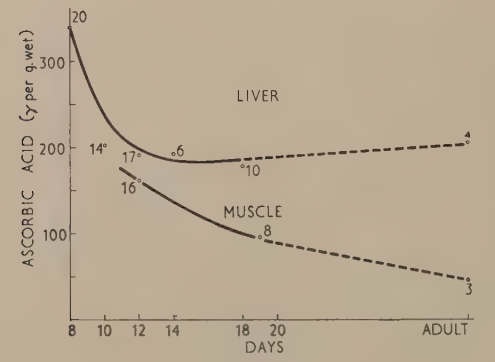
The technical obstacles to making reliable estimations on individual organs at the early stages of development are even greater than with whole embryos, and therefore no values are given before the 8th day of incubation. The results obtained with brain, heart, skeletal muscle, liver, and intestine are shown in Text-figs. 6, 7, and 8. The general trend is for the concentration of ASA to fall



TEXT-FIG. 6. ASA concentration in brain of chick embryo.



TEXT-FIG. 7. ASA concentration in intestine and heart of chick embryo.



TEXT-FIG. 8. ASA concentration in liver and muscle of chick embryo.

with age, particularly when the embryonic values are compared with those obtained in the organs of the adult cock. The fall in ASA from embryonic to adult life was most marked in brain and muscle. The ASA concentration in the embryonic liver decreased substantially from the 8th to the 10th day, and thereafter it remained approximately at the adult level, while the intestine showed a slightly higher concentration in the adult bird than in the embryo.

Measurable amounts of ASA appear in the yolk sac at the later stages (Table 3). This may have been either synthesized or filtered back by the sac villi.

TABLE 3

ASA concentration in the yolk sac

Days of incubation	*Reduced ASA (γ/g.)*
16	14
17	19
18	18
19	23
21	19

The ratio of oxidized/reduced vitamin in the whole embryo increased with age. During the first week the percentage of oxidized vitamin oscillated between 0 and 4 per cent. of the 'total' vitamin estimated by Bolin & Book's method, while at the 18th and 19th days as much as 29–33 per cent. was extracted in the oxidized forms (dehydroascorbic plus diketogulonic acid). This was probably due to the increase in the blood-volume of the embryo, since a large proportion of the circulating vitamin is usually extracted in the oxidized state because of its interaction with oxyhaemoglobin (Butler & Cushmann, 1940; Roe & Kuether, 1943). Individual organs of late embryos and of adult birds drained of blood contained negligible proportions of oxidized vitamin, in accordance with the experience of various workers on other animal species.

DISCUSSION

The contention of Ray (1934) that ASA is absent until the 4th day of incubation must be attributed to the low sensitivity of the methods then available, since at least a hundred embryos would be necessary to obtain a clear reading with the visual titration method at the 3rd day of incubation. Barnett & Bourne (1942) also failed to find any ASA in the embryo before the 4th day with a histochemical test based on the reduction of silver nitrate, but Weel (1948), using Bourne's method, obtained a positive reaction in the limb-bud at the 3rd day.

The vitamin content per embryo ceases to increase at the 16th day, coinciding with the inflexion of the weight curve. The evolution of CO_2 per egg also becomes stable at this date according to the figures of Pott & Preyer and of Bohr and Hasselbalch quoted by Needham (1931).

The concentration of ASA per gram of wet and dry tissue is highest at the beginning of development coinciding with the maximum specific growth rate and shows a hump towards the middle of development, between the 7th and the 12th day, which could be related to any of a number of important metabolic events which take place at this stage, namely, a peak in the metabolic rate and in the ratio of burnt to stored solids, a change-over from carbohydrate to fat consumption accompanied by a peak in the lipase activity of the yolk sac, a peak in free glucose and the onset of glycogen storage and insulin secretion (refs. in Needham, 1931, 1942). It is noteworthy in this connexion that ASA catalyses the oxidation of some fatty acids and lipids and that lipase activity is decreased in scurvy (refs. in Reid, 1954). Insulin secretion also falls steeply in scorbutic guinea-pigs (Bannerjee, 1944). A correlation between general metabolic activity and ASA concentration has also been found by Bessey & King (1933) in animals and by others in plants (refs. in Mapson, 1953).

The heterauxetic constant found for ASA in the embryo is almost identical with that obtained by others for glutathione (GSH) (data in Needham, 1942). Needham's (1942) cautious remark that 'it is doubtful whether ascorbic acid shows the bradyauxesis of the other highly reducing substance, glutathione' reflects the lack of sufficient and accurate estimations at the time. Early in his work on GSH, Hopkins (1921) found that this compound was missing from the white and yolk of the egg, but the chick blastoderm gave a positive nitroprusside test. This suggests that ASA and GSH appear simultaneously in the embryo from the beginning of development. ASA and GSH also appear simultaneously in germinating seeds and sprouting tubers (see Mapson, 1953).

The curve of GSH concentration per unit of dry weight obtained by Murray (1926) in the chick embryo overlaps closely with the corresponding portion of the ASA concentration curve (Text-fig. 5). The relationship between these two compounds is of interest because of the protective action of GSH on ASA, the possible occurrence in animal tissues of a respiratory pathway (Ames & Elvehjem, 1946) like that described by Mapson & Goddard (1951) in plant tissues, and the role assigned to SH groups in cell-division by Rapkine and others (discussion in Needham, 1942; Swann, 1957).

The results obtained with isolated organs confirm the findings of Bessey & King (1933) that the organs of rapidly growing animals show higher vitamin titres than those of older individuals. The values given by these authors for the adult fowl are in good agreement with the present figures. The steepest fall in ASA with age was found in the two tissues which cease to divide in adult life, namely, brain and muscle. On the other hand, the intestine, which keeps its embryonic level of ASA in postnatal life, has a high mitotic rate and a fast turnover of DNA in the adult organism (Hevesy & Ottesen, 1943).

The stability of ASA concentration in the liver after the 10th day agrees with the work of Ray (1934), who found steady values around 270 γ per g. wet weight in late development. The decrease in liver ASA from the 8th to the 10th day

found in the present experiments may be significant in connexion with the onset of glycogen storage in this organ, which takes place approximately at the 8th day (refs. in Needham, 1942). Barnett & Bourne (1942), using Bourne's histochemical silver method, could locate ASA in the liver up to the 10th day, but found 'none at all' after that date. ASA appears to influence glycogen storage and synthesis (see Reid, 1954), while ASA deficiency causes a marked decrease in liver phosphorylase (Murray, 1950).

According to Olivo & Porta (1931) the mitotic index of the liver falls from 14·3 to 7·73 from the 7th to the 10th day, which represents a decline of about 45 per cent., and is therefore approximately equivalent to the fall in ASA concentration for the same period. Here, however, the correlation ends, for the vitamin concentration remains constant after the 10th day, while the mitotic index continues to fall to about 1·0 at hatching. On the other hand, the specific growth rate $(1/w)\,(dw/dt)$ calculated from Olivo & Porta's figures falls only from about 0·8 to about 0·2 from the 7th to the 19th day. It may be noted that this is a less subjective and more accurate index of growth than the mitotic coefficient.

Similarly, there is no visible correlation between the pronounced fall in the mitotic index of the heart, which decreases nearly fifteen times from the 7th to the 21st day (Olivo & Porta, 1931), and the moderate fall in its ASA concentration.

Since no direct stimulation of cell-division by ASA has been definitely demonstrated in animal tissues and ASA is not a growth factor for most micro-organisms and protozoa, its marked influence on the growth of guinea-pigs and its active synthesis by rapidly growing tissues may perhaps be connected with the building of intercellular materials rather than with cell-growth and multiplication. It is now well established that ASA is necessary for the synthesis and maturation of reticulin, collagen, the bone matrix, and dentine, and there is good evidence that it also influences the metabolism of mucopolysaccharides (see review by Reid, 1954). Yet, in spite of the fact that most of the functions of ASA so far established are exerted on mesenchymal structures, epithelial and nervous tissues are richer in ASA than mesenchymal tissues in both the embryo and the adult.

It cannot be established from the present results whether the vitamin can be synthesized by all or only by some tissues of the embryo, nor whether the variations in ASA content at different sites and at different stages of development are due to variations in synthetic capacity or merely to differential storage. It is possible that the ability to synthesize ASA shown by the early embryo is not shared by all tissues in late development and that some cells may lose the synthetic mechanism in the course of differentiation.

SUMMARY AND CONCLUSIONS

Daily weighings of chick embryos of the Light Sussex strain gave a sigmoid curve with a point of inflexion at the 16th day. It was shown that inclusion of

the 'spare yolk' after the 19th day may give a misleading impression of fast growth at the end of development. The growth rate was greatest at the beginning of development and the specific growth rate decreased approximately as an inverted hyperbola with time.

No ascorbic acid was found in the unincubated egg, but it appeared in the blastoderm from the beginning of development coinciding with the appearance of glutathione. The total amount of vitamin per embryo gave a sigmoid curve with a maximum at the 16th day coinciding with the peak of the differential growth curve. The concentration of ASA per unit weight, however, like the specific growth rate, was maximal at the beginning of development; it showed a lower peak towards the 10th day which was not correlated with any significant variation in growth, although it may have been related to any of several metabolic changes that occur at this stage in the chick embryo.

ASA estimations in individual organs showed that, in general, epithelial tissues are richer in vitamin than mesenchymal tissues, and that in those tissues that divide very actively during embryonic development but cease to multiply in adult life, such as muscle and the central nervous system, the vitamin C concentration drops markedly with age. The brain showed a remarkably high concentration in early development.

It may be concluded that the changes in weight and in ASA content of the chick embryo both follow sigmoid curves, that the chick embryo is able to synthesize ASA from early stages of development, and that there is some correlation between the concentration of ASA and the rate of growth both in the whole embryo and in some of its organs.

REFERENCES

Ames, S. R., & Elvehjem, C. A. (1946). Enzymatic oxidation of glutathione. II. Studies on the addition of several cofactors. *Arch. Biochem.* **10**, 443–53.

Bannerjee, S. (1944). Vitamin-C and carbohydrate metabolism. IV. Effect of vitamin-C on the insulin content of guinea-pig pancreas. *Ann. Biochem.* **4**, 33–36.

Barnett, S. A., & Bourne, G. (1942). Distribution of ascorbic acid (vitamin C) in cells and tissues of the developing chick. *Quart. J. micr. Sci.* **83**, 259–98.

Bessey, O. A., & King, C. G. (1933). Distribution of vitamin C in plant and animal tissues, and its determination. *J. biol. Chem.* **103**, 687–98.

Birch, T. W., Harris, L. J., & Ray, S. N. (1933). A micro-chemical method for determining the hexuronic acid (vitamin C) content of foodstuffs, etc. *Biochem. J.* **27**, 590–4.

Bolin, D. W., & Book, L. (1947). Oxidation of ascorbic acid to dehydroascorbic acid. *Science*, **106**, 451.

Butler, A. M., & Cushman, M. (1940). Distribution of ascorbic acid in the blood and its nutritional significance. *J. clin. Invest.* **19**, 459–67.

Harris, L. J., & Mapson, L. W. (1947). Determination of ascorbic acid in presence of interfering substances by the 'continuous flow' method. *Brit. J. Nutr.* **1**, 7–38.

—— —— Kodicek, E., Moore, T., & Booth, V. (1948). Chemical methods for the estimation of vitamins. *Food Manuf.* **23**, 316–22.

Hevesy, G., & Ottesen, J. (1943). Rate of formation of nucleic acid in the organs of the rat. *Acta physiol. scand.* **5**, 237–47.

Hopkins, F. G. (1921). On an autoxidisable constituent of the cell. *Biochem. J.* **15**, 286–305.

Huxley, J. S. (1932). *Problems of Relative Growth.* London: Methuen.

Mapson, L. W. (1953). Function of ascorbic acid in plants. *Vitam. & Horm.* **11**, 1–28.

MAPSON, L. W., & GODDARD, D. R. (1951). The reduction of glutathione by plant tissues. *Biochem. J.* **49**, 592–601.

MURRAY, H. A. (1926). Physiological ontogeny. A. Chicken embryos. IX. The iodine reaction for the quantitative determination of glutathione in the tissues as a function of age. *J. gen. Physiol.* **9**, 621.

MURRAY, H. C. (1950). Activity of some enzymes involved in carbohydrate metabolism in normal and scorbutic guinea-pigs. *Proc. Soc. exp. Biol. Med., N.Y.* **75**, 598–601.

NEEDHAM, J. (1931). *Chemical Embryology*. Cambridge University Press.

—— (1942). *Biochemistry and Morphogenesis*. Cambridge University Press.

OLIVO, O. M., & PORTA, E. (1931). Differenze nell' accrescimento ponderale, coefficiente mitótico dell'accrescimento e durata della mitosi tra fegato e cuore embrionali di pollo. *Monitore zool. ital., suppl.* **41**, 213–18.

RAY, S. N. (1934). A note on the presence of vitamin C in the chick embryo. *Biochem. J.* **28**, 189–91.

REID, M. E. (1954). Ascorbic acid. VIII. Effects of deficiency in animals. In *The Vitamins*, pp. 269–347, ed. Sebrell & Harris. New York: Academic Press.

ROE, J. H., & KUETHER, C. A. (1943). The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. biol. Chem.* **147**, 399–407.

—— & OESTERLING, M. J. (1944). The determination of dehydroascorbic acid in plant tissues by the 2,4-dinitrophenylhydrazine method. *J. biol. Chem.* **152**, 511–17.

SWANN, M. M. (1957). The control of cell division: a review. I. General mechanisms. *Cancer Res.* **17**, 727–57.

WEEL, VAN P. B. (1948). Histophysiology of the limb-bud of fowl during its early development. *J. Anat., Lond.* **82**, 49–57.

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